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INVESTIGATION OF PHENOL RESIDUES IN HONEY AND BEESWAX

by

PATRICIA A. DAHARU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INVESTIGATION OF PHENOL RESIDUES IN HONEY AND BEESWAX submitted by PATRICIA A. DAHARU in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in FOOD CHEMISTRY.

DEDICATION

To My Parents

"Courage is the first of human qualities because it is the quality which guarantees all others."

Winston Churchill

ABSTRACT

Analysis for residue levels of the bee repellent, phenol, in honey revealed that phenol (carbolic acid) is widely used in Alberta. The results indicated that 40 out of 67 samples of honey examined from Alberta contained phenol levels varying from 1 ppm to 11.1 ppm, with an average of 7.4 ppm. Phenol residues were identified in honey samples obtained from other provinces in Canada, the United States and Mexico. Practical investigation conducted at hives located near Edmonton, Alberta by application of phenol on a phenol board (acid board) showed that all honey collected contained residues of phenol, which are impossible to remove without adversely affecting the honey flavor. Beekeepers can limit phenol residues in honey by minimizing hive exposure to the phenol board and collecting a higher proportion of capped honey. Phenol was also found in beeswax samples. Honey examined prior to exposure to the phenol board contained phenol. This finding, substantiated by analysis of wax samples, implied the transmission of phenol residues by the reuse of empty combs, a method commonly practised by beekeepers in the seasonal collection of honey. Discontinuation of the use of phenol as a bee repellent was observed to result in a reduction of phenol residues to trace levels (0.5 ppm). The detection of the medicinal taste of phenol in honey by an untrained panel appeared to be at levels of about 20 ppm, at least ten times higher than the detection level of phenol in water. The method recommended for phenol analysis in honey is the high pressure liquid chromatographic (HPLC) method. For beeswax, the Gibbs or aminoantipyrine method, adapted to the determination of phenol in beeswax, is highly suitable.

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1. INTRODUCTION

Honeybees (Apis mellifera L.) have fascinated mankind from the beginning of times. Early historical reports reveal picturesque myths associated with honey and beliefs in the curative properties of honey which still persist in many parts of the world today. Honey is a unique food and is valued by many as one of Nature's most magnificent achievements because of the flavor, aroma and extraordinary properties associated with it. Excellent reviews of the early history are presented by More (1976) and Crane (1975). Dionyssus (the Greek Bacchus) is depicted as a god of the honey-drink, mead and not wine.

The world production of honey is estimated at 884,300 metric tonnes for 1981 (Anon., 1982) and the figure represents an increase over the preceding five years. The production of honey in both Alberta and Canada reached record levels in 1981. The 1981 figure quoted for honey production (Boyce, 1982) in Alberta is 10,478 metric tonnes and represents about one third the total production of Canada. It is of interest to note that the province of Alberta is the largest honey producer in Canada. The export markets for Canadian honey include chiefly, the United States, the European Economic Community (EEC), Japan, Sweden and the Caribbean. The most significant developments affecting the export production of honey in Canada for 1981 and 1982 are the record Chinese crop and the second successive poor crop in the United States. Canadian honeys also have to compete in price and quality with honey from China, Argentina and Mexico, the major honey producers and exporters.

The use of phenol or carbolic acid as a bee repellent began in the 1930's (Anon., 1968). A concentrated solution of phenol is applied to the adsorbent cloth on the underside of a special hive cover called a phenol board. The phenol board is placed on the top of the hive and the heat from the sun vaporizes the phenol, which then has a repelling effect on the bees, therefore enabling the beekeeper to collect honey and/or beeswax with a minimum of interference. However, the use of phenol is of great concern because of possible contamination. Significantly high residues of phenol impart a disagreeable

medicinal taste to the honey. Efforts were made to find a substitute for phenol because of possible tainting of the flavor of honey and also, because of implications regarding the toxicity of phenol. Many chemicals were evaluated as potential bee repellents (Woodrow *et al.*, 1965), but few were found to be suitable. Benzaldehyde, propionic anhydride, and butyric anhydride were found to be effective. In addition, a mechanical device, a bee blower was invented. However, at present, phenol is still used on a large scale as a bee repellent.

One of the important objectives of the project was to monitor levels of phenol residues from honey produced in Alberta. Honey samples from outside Alberta were also studied and the results presented. Practical investigation of factors affecting phenol residue levels of honey was conducted by application of phenol on a phenol board and analysing the collected samples of honey and wax.

Methods of analysis of phenol were adapted for determination of phenol in both honey and wax. The HPLC method on a normal phase column reported by Sporns (1981) for the analysis of phenol residues in honey was used and in addition several techniques were formulated. The techniques described include an HPLC method on a reverse phase column, colorimetric tests incorporating the use of the Gibbs reagent and the aminoantipyrine reagent, and a fluorometric technique.

An attempt at sensory evaluation using an untrained panel was carried out to determine a flavor threshold of phenol in honey.

Phenol is not recommended by the United States Department of Agriculture (USDA) and by Canadian authorities for use as a bee repellent because of possible contamination of honey due to careless use of phenol. Recent communication indicates that regulations may be formulated in the near future restricting the use of phenol as a bee repellent. Thus, it may be essential to have procedures to quantitate phenol residues in honey, in addition to the existing standard tests which are specified by European Economic Community countries. The tests prepared by the Codex Alimentarius Commission of the Joint FAO/WHO Food Standards Programs (the Food and Agriculture

Organization and World Health Organization of the United Nations) include monitoring of color, ash, sugar composition, moisture levels, freedom from particulate matter, levels of enzymes such as diastase, and hydroxymethylfurfural content. A fast and reliable method suitable for routine analysis in quality control laboratories is described for dectection of phenol residues in honey by the reverse phase HPLC method. The colorimetric methods are recommended for the analysis of wax, which is also essential to quantitate since wax absorbs a greater amount of phenol than honey does.

2. REVIEW OF LITERATURE

2.1 Methods of Collecting Honey

Four methods of removing bees from honey supers will be discussed. They involve use of bee repellents, the shake-and-brush method, use of bee-escape boards and bee blowers.

2.1.1 Bee Repellents: General Overview

Bee repellents are used to facilitate the removal of bees from honey supers, thus enabling the beekeeper to harvest honey with a minimum of disruption. Bee repellents are widely used in most large scale commercial operations in Alberta and elsewhere. The efficacy of bee repellents in controlling the behavior of the bees and the resulting reduction in labor cost because unskilled labor can be used and honey rapidly collected, are two important factors responsible for the widespread use in many countries.

In addition to bee repellents for honey collection, bee repellents have been investigated for the prevention of bee losses from insecticides applied to flowering plants. The development of suitable repellents to protect individuals hypersensitive to stings would also be highly desirable.

The chemicals commonly used as bee repellents for collection of honey are phenol, benzaldehyde, propionic anhydride and butyric anhydride commonly sold under the brand name Bee-Go.

2.1.1.1 Phenol

Phenol is used in many countries including Canada, the United States, Mexico, Australia, New Zealand and South Africa.

A concentrated solution of phenol (up to 90%) is made by dissolving phenol crystals in water and sprinkling the solution evenly on the undersurface of a phenol board which is lined with adsorbent cloth. The phenol board or acid board consists of a frame with the same dimensions as the hive cover and a rim 3-5 cm deep. On the upper surface of the phenol board is a black sheet of metal in direct contact with the cloth. The phenol board with the

solution added, is then placed on top of the hive with the black surface uppermost (Plate 1). The heat from the sun is absorbed by the black cover of the phenol board and vaporizes the phenol, which then has a repelling effect and forces the bees down to the lower supers. The uppermost honey super is removed and the phenol board is placed on the next lower honey super of the hive. By successive removal of the uppermost super and placing of the phenol board on the next lower super the honey supers can be removed in rapid succession.

Usually, beekeepers apply smoke to start the bees moving and also to "mask" the scent of crushed bees. The gland of the sting chamber produces isopentyl acetate, an alarm pheromone, which is released from the crushed bees and incites aggressive behavior among bees. Thus, the bee smoker is an indispensable tool and is shown in Plate 2. Some important tools used in beekeeping are illustrated in Plate 3.

The varying degrees of success in the use of phenol with the phenol board, concern about handling of the chemical since it can cause serious burns, its ineffectiveness on cool days as a repellent, and fears of tainting or contamination of the honey resulted in the search for other chemical repellents.

2.1.1.2 Acid Anhydrides

Propionic anhydride was developed by Woodrow and Moeller of the United States Department of Agriculture to replace phenol as a bee repellent (Anon., 1961). Propionic anhydride hydrolyses with moisture from the air to slowly form propionic acid (Figure 1). Propionic anhydride is applied to an adsorbent pad and placed in a special fume chamber fitted over the top of an empty super on top of the hive. Fumes of the anhydride are pumped with a smoker bellows and are distributed evenly through holes in a dispersion board located in the fume chamber (Walsh, 1965; Anon., 1961; Anon., 1968). Propionic anhydride works on warm and cool days and reports indicate that the bees remain "gentle". However, the chemical has not been effective as a repellent under all conditions. For example, beekeepers indicated the anhydride to be effective on North Island, New Zealand, but completely ineffective on the

Plate 1

Phenol boards placed on hives with upper black surface exposed

Plate 2

Bee Smoker (Top)

Smoking of bees in the process of collecting individual frames from honey supers (Bottom)

Plate 3

Equipment consisting of a bee brush, uncapping fork, and hive tool (Top)

Scraping of surface capping of wax from a capped frame (Bottom)

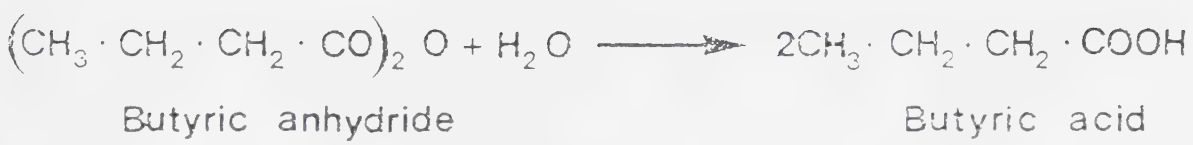


Figure 1. Equations showing hydrolysis of the acid anhydrides to the corresponding acids.

South Island (Walsh, 1965).

Butyric anhydride works in a similar manner to that of propionic anhydride and is hydrolysed to butyric acid (Figure 1).

Reich *et al.* (1969) attributed the repellent effect to the vapors of the acids formed on hydrolysis of the acid anhydrides. Measurements of the vapor pressures of the acids lie between 0.25 to about 1.5 millimeters of mercury at 20°C. Reich *et al.* (1969) observed that the vapors of organic acids and bases which have a pK of 1 to 8 were irritating to bees. They postulated that the contact of the irritating vapors with moist membranes of the bees, for example the eyes, provided the irritating factor. However, moist membranes in insects are almost always internal and the eyes of bees are not covered with moist membranes. The respiratory system of insects terminates in "stellate" cells from which fine tubules lead to individual cells. It is probable that the vapors penetrate into the tubules which contain fluid. Another possibility is that the acid vapors come in contact with chemosensory cells in hairs on the surface of the body. The surface of these dendrites and the pore at the end of the sensillum (hair) are covered with a viscuous fluid which is as close to a "moist membrane" as can be found outside of the insect (Gooding, 1983).

2.1.1.3 Benzaldehyde

Benzaldehyde, also known as artificial oil of almonds, was introduced by Townsend (1963). A solution of benzaldehyde applied to the undersurface of the phenol board was reported to repel the bees efficiently. Field trials with benzaldehyde revealed that air temperatures above 27°C resulted in "stupefaction" of the bees and thus failure to drive the bees from the honey supers.

Benzaldehyde is generally recommended as a good repellent since it is effective between 15-27°C under cloudy or sunny conditions. Measures recommended in the use of benzaldehyde include the addition of insulating material over the phenol board and smoking of the colony before placing the board on the hive. Townsend (1963) and Walsh (1965) both claim residues of benzoic acid are formed in honey by oxidation of benzaldehyde. They believe the presence of residues of benzoic acid is not as serious as residues of

other bee repellents, such as phenol, since benzoic acid is permitted for use as a food preservative. The structure of benzaldehyde, which is similar to phenol is illustrated in Figure 2.

2.1.1.4 Other Aspects of Bee Repellents

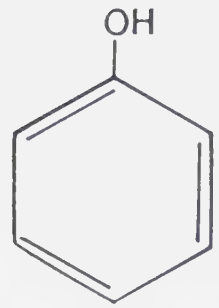
Natural and synthetic compounds were screened as attractants and repellents of the honey bee, Apis mellifera by observation of the responses of bees to the vapors of a variety of compounds (Woodrow *et al.*, 1965). It was found that the repellents elicited more constant responses than the attractants. The most potent repellents were the amines, acids or acid anhydrides and carbonyl compounds. The weak to moderate repellents included phenols, alcohols, esters and ethers. Woodrow *et al.* (1965) rated propionic anhydride, propionic and acetic acids as highly repellent, nonpersistent and causing no contamination. Phenol was rated as weak to moderately repellent.

In contrast to the observations made by Reich *et al.* (1969), Townsend (1963a) reported that the perception of repellents was through odor. Experiments performed by removal of the bees' antennae showed a lack of response to the compound. Townsend claims the repellents affect the bees through chemoreceptors on the flagellum of the antenna, and not by irritation of the respiratory system.

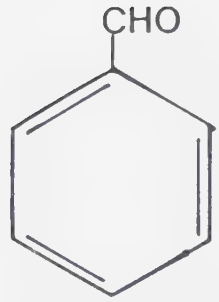
Field experiments conducted on a number of repellents indicate varying degrees of success under different weather conditions. Also, the response by bees to repellents changes under different situations as demonstrated by variations in the amount of smoke required to control a colony of bees at intervals. Thus, further investigation is needed to find the ideal repellent since a third factor, the human element must be accounted for, to adjust conditions and estimate when the bees are repelled.

2.1.2 The Shake-and-Brush Method

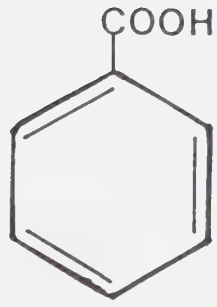
The method is used by beekeepers with a small number of hives and requires a minimum of apparatus, either a goose-wing or bee brush. A few puffs of smoke are directed over the frames of the honey super and each



Phenol



Benzaldehyde



Benzoic Acid

Figure 2. Structures of phenol, benzaldehyde and benzoic acid.

frame containing honey is removed and the bees brushed (Plate 4). The shake-and-brush method is time consuming.

2.1.3 Use of the Bee-Escape Boards

Bee-escapes are small metallic devices which are fitted in a wooden or plywood board. There are various types of bee-escapes, however, they all contain light springs, which are arranged to open in one direction (Figure 3) so that bees can leave the honey super and move down to the lower super or brood chambers below, but cannot re-enter the super. Plate 5 shows two bee-escapes on an escape board.

The bee-escape boards are placed for 24 or 48 hours under the honey supers to be collected. Usually, no more than two supers of honey are removed at once. Care must be taken to ensure that the honey super is bee-tight to avoid robbing of the honey. This method is very effective, but may fail if the springs of the escape become stuck, for example with a drone, or if propolis is glued over the springs by the bees. At least two trips are required to remove the honey, the first, to place the escape board and the second to remove it after the bees leave the honey super. Also, usually about a day of honey production is lost waiting for bees to clear the supers containing honey.

2.1.4 Use of Bee Blowers

This method involves initial smoking of the hive and placing the super on a stand. A jet of air is directed at the frames in the super and the bees are blown out. This method is recommended by the USDA because it is efficient under all conditions of temperature, fast and guarantees no contamination of the honey collected. One model of bee blower is shown in Plate 6. An improvement in the design of bee blowers to minimise the noise level will undoubtedly aid in increasing its use.

Plate 4

Honey super containing individual frames (Top)

Shake and brush method of collecting honey demonstrated by removing individual frames and brushing bees (Bottom)

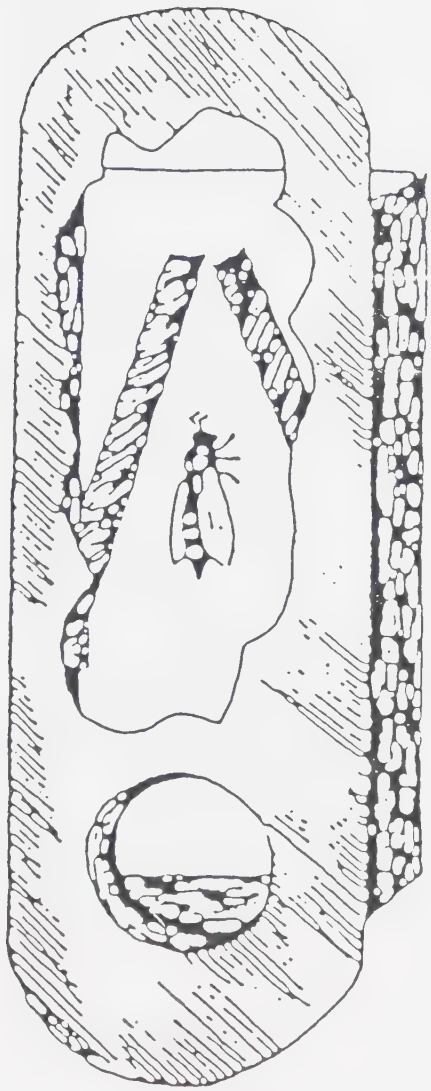


Figure 3. Porter bee-escape (More, 1976).

Plate 5

Bee escapes inserted in an escape board (one of outer bee escapes removed and placed in middle of escape board)

Plate 6

Bee blower

2.2 Sources and Uses of Phenol

Phenol was first isolated from coal tar by the process of fractional distillation. Synthetic processes developed for the production of phenol include fusion of sodium benzene sulfonate with sodium hydroxide, and heating of monochlorobenzene with aqueous sodium hydroxide under high pressure.

A review of the literature reveals the extensive uses of phenol (Liao and Oehme, 1980a). Some of the uses are in the manufacture of explosives, fertilizers, wood preservatives, textiles, perfumes, rubber, plastics, drugs, pharmaceutical preparations. Other uses are in the petroleum, leather, paper, soap, toy, tanning, dye, and agricultural industries (Deichmann and Keplinger, 1963). Phenol is also employed as a disinfectant in germicidal paints and as a slimicide.

Phenol was first used by Lister in 1867 for aseptic surgery and generally as an antiseptic. Early uses of phenol which were extensive and popular for a number of years resulted in cases of chronic or subacute phenol poisoning. The medical uses of phenol are now limited, but there are many common products which contain phenol. Phenol is used as an ingredient in preparations for cold sores, chapped lips (Blistex, 0.4%), in sorethroat gargles (Chloraseptic, 1.4%; Cepastat, 1.4%), toothache preparations (Anbesol, 0.45%), and ear wax drops. Many other uses include treatment of skin lesions, althlete's foot and fungal diseases, also application as a topical anesthetic for pruritic lesions (1% lotion or ointment).

2.3 Toxicity of Phenol

Phenol, either as a solution or as a vapor, has been reported to readily penetrate intact skin. Animal studies have indicated that the extent of percutaneous absorption of phenol depends on the area of skin exposed, rather than the concentration of the applied solution (Deichmann and Keplinger, 1963). Evidence suggests that in man phenol may be considerably less toxic by ingestion than by absorption through the skin (Gosselin *et al.*, 1976). In concentrations of 10 to 40%, phenol is reported to be a destructive agent to

the skin (keratolytic agent) and nerves (neurolytic agent).

Studies have shown phenol to be a cancer promoter. That is, an application of phenol after exposure to a genotoxic carcinogen results in an increase in tumor response. Promoters, although believed to be involved in human cancer, are highly dose dependent and require the presence of the agent for a long time (Weisburger and Williams, 1979). Wattenberg *et al.* (1976) reports that phenol is not a cocarcinogen and, as shown for many phenolic promotors, these compounds can actually have an inhibitory effect, if applied before or during application of a genotoxic carcinogen.

A current review of over-the-counter (OTC) drugs by the United States Food and Drug Administration (FDA) reports that phenol is safe for application to oral mucous membranes in concentrations up to 1.5% in aqueous solution. A 1.5% concentration of phenol in 20% ethyl alchol is used as a dental rinse, whereas a 1.5% concentration of phenol in 70% ethyl alcohol is used for direct application to gums. The specified concentration of phenol is the maximum generally recognized as safe (GRAS) concentration with a limit of 7 days use for any course of therapy, unless treatment is supervised by a dentist or physician (Federal Register, 1982c). The same Panel also evaluated reports of the carcinogenic and cocarcinogenic potential of phenol and phenolic substances. A summary (Federal Register, 1982c) indicates that the cocarcinogenic effect of phenolic compounds is reversible and low concentrations by themselves are not carcinogenic.

Results from studies of metabolism and excretion of phenol from animals, including man, have shown that phenol is rapidly absorbed, metabolized and excreted from the body. The conjugation of phenol to the glucuronide, sulfate and hydroxylated derivatives are shown in Figures 4, 5 and 6. In man, 90% of a non-toxic oral dose (0.01 mg/kg) of [^{14}C]-labelled phenol was excreted in 24 hours, principally as the sulfate and as the glucuronide, with small amounts of sulfate and glucuronide conjugates of a metabolite, hydroquinone (Capel *et al.*,1972). It has been reported that larger doses of administered phenol can result in free phenol in the urine.

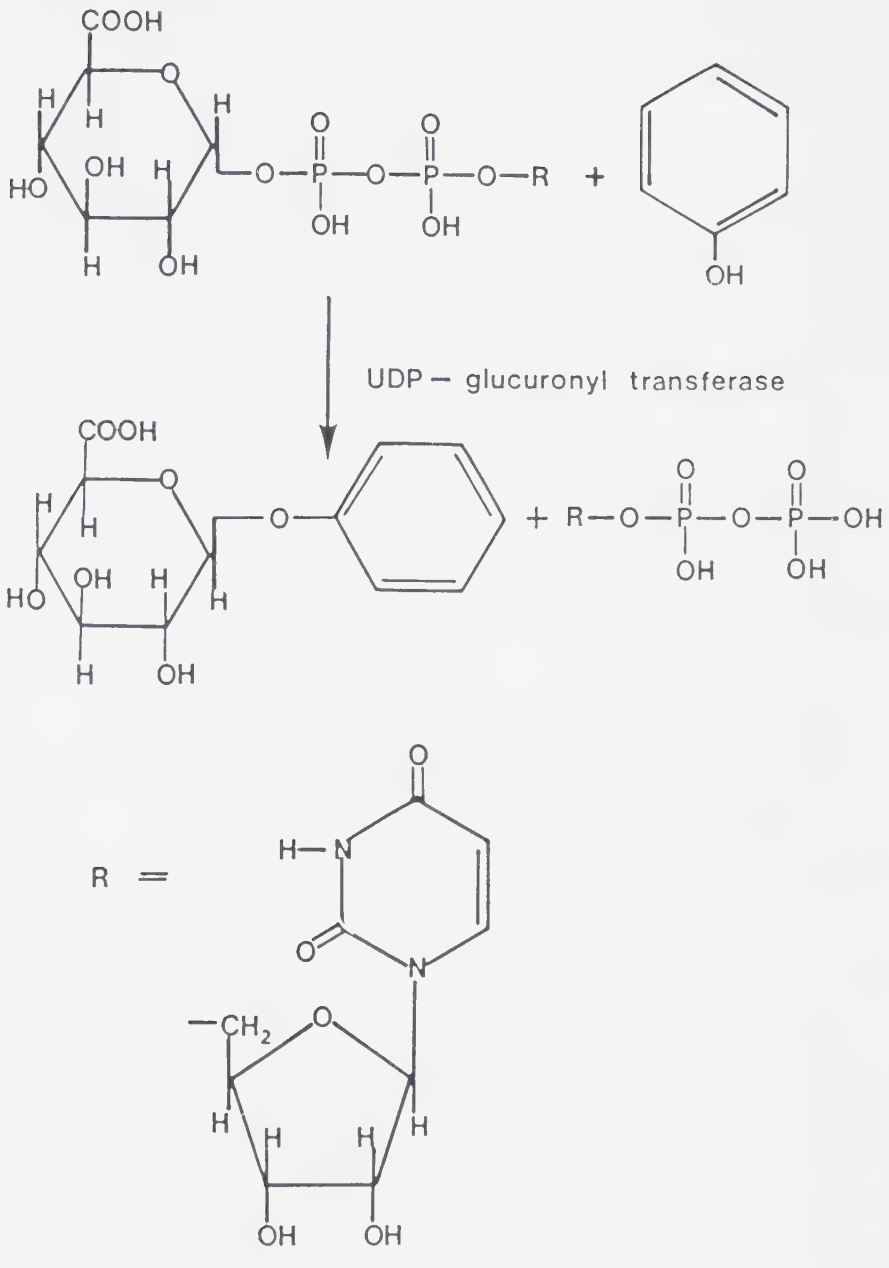


Figure 4. Conjugation reaction of phenol with uridine-5'-diphospho -D-glucuronic acid (UDPGA) to form uridine-5'-diphosphate (UDP) and the phenol glucuronide according to Neal (1980).

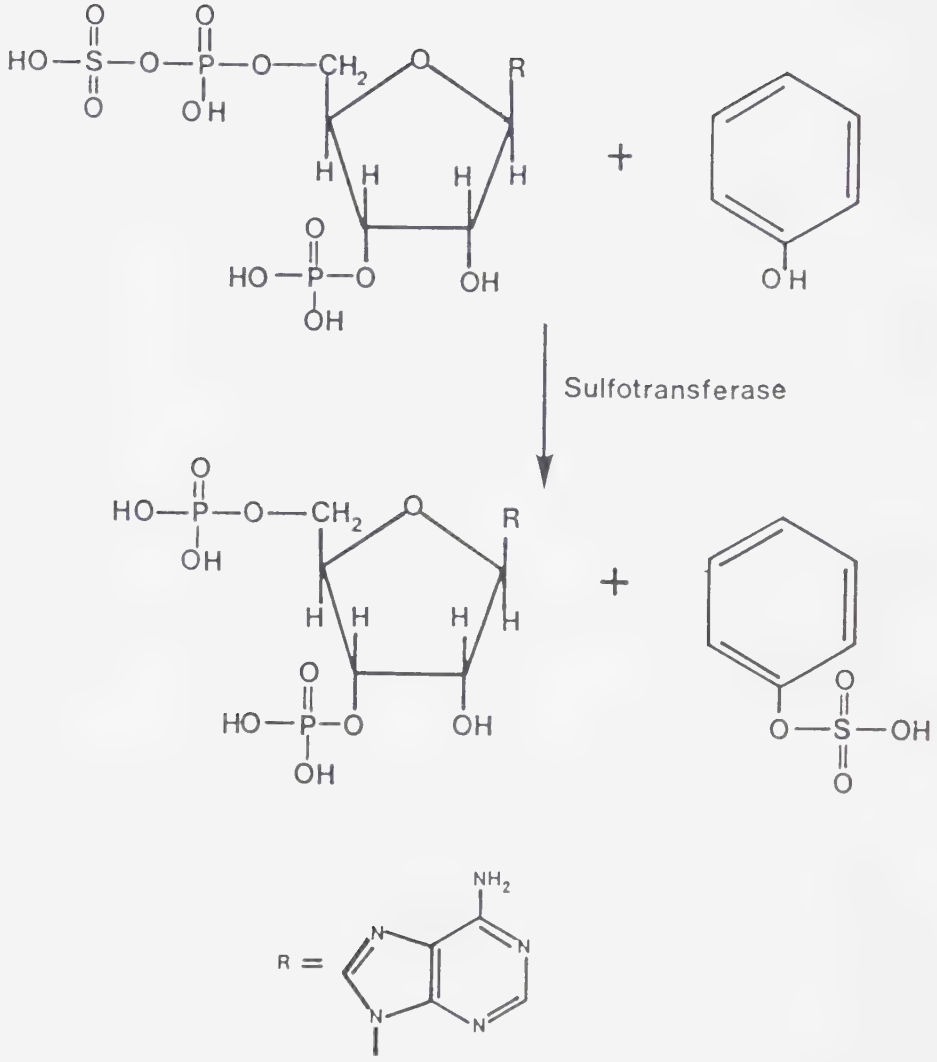


Figure 5. Conjugation reaction of phenol with 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to form 3′-phosphoadenosine-5′-phosphate (PAP) and the sulfate ester according to Neal (1980).

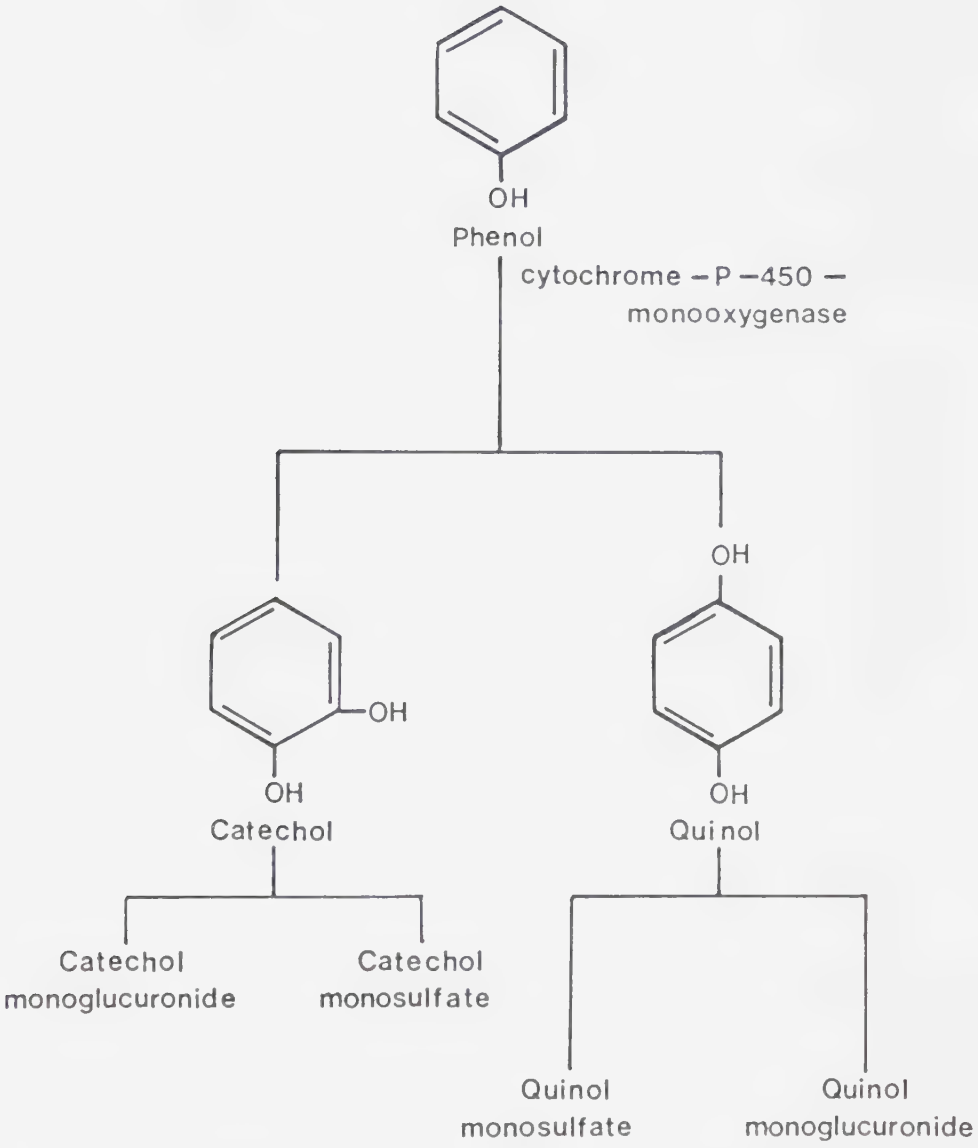


Figure 6. Hydroxylation of phenol and biotransformation to the glucuronide and sulfate derivatives according to Reeves (1981).

It should be emphasized that in general the metabolism of a compound is not fixed and phenol may be excreted in different forms by the same individual. Biotransformations vary with factors such as sex, age, genetic constitution and amount of compound ingested. An important principle in detoxification is a conjugation reaction in which a more water-soluble derivative is synthesized and excreted in the urine. Renal excretion is in fact the principal route of elimination of phenol.

In the biotransformations illustrated in Figures 4 and 5 the cofactors are uridine-5'-diphosphate-D-glucuronic acid (UDPGA) and 3'-phospho- adenosine-5'-phosphosulfate (PAPS). The enzymes, uridine-5'-diphosphate-glucuronyl transferase (UDP-glucuronyl transferase), sulfotransferase, and cytochrome-P-450-monooxygenase are located mainly in the liver, kidney and intestine. During the biotransformation of phenol to the phenol glucuronide by the UDP-glucuronyl transferase enzyme there is inversion of configuration. The glucuronide derivative with phenol has a β configuration.

It has been stated that glucuronidation of phenol is more important than sulfate conjugation in animals because of greater availability of UDPGA over PAPS (Neal, 1980).

2.4 Analytical Methods for Quantitation of Phenol

2.4.1 Extraction Procedures

In the following discussion phenol and other phenolic compounds are referred to as phenols.

Isolation of phenols from material has been achieved by techniques of solvent extraction and/or steam distillation. Steam distillation has been employed for the isolation of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), phenolic antioxidants added to dehydrated potato samples (Filipic and Ogg, 1960; Sloman *et al.*, 1962). It is also used for extraction procedures for phenols in waste water. Sporns (1980), who first described an HPLC methodology for determining phenol residues in honey, utilized the process of

steam distillation in the presence of an internal standard.

Steam distillation is applied to liquids which are completely immiscible or liquids which are miscible to a slight extent.

In steam distillation the boiling point at which the mixture distills is attained when the sum of the vapor pressures of the components present is equal to that of the atmosphere. Steam distillation at atmospheric pressure results in separation of the immiscible higher boiling component at a temperature below 100°C, the boiling point of water. The use of steam distillation is of considerable value since water has a low molecular weight and a comparatively moderate vapor pressure, therefore allowing isolation of large amounts (weight) of substances of high molecular weight and low vapor pressures.

A method of increasing the volatility of phenol by the addition of solutes has been reported (Norwitz and Keliher, 1982). The authors found that the addition of 15 g of sodium chloride to a 25 mL sample followed by steam distillation resulted in optimum recovery of the phenol in the first few milliliters of distillate. Recoveries up to 25 mg of phenol per 25 mL were recorded. The addition of sodium chloride to obtain a quantitative distillation of phenol has been attributed, not to an increase in boiling point caused by the solute, but to a mechanism in which the amount of water available as a solvent for the phenol is depleted by the hydration of sodium and chloride ions.

2.4.2 Determination Procedures

Quantitation of phenol and phenolic compounds has been accomplished by paper chromatography, spectrophotometry, thin layer chromatography, gas–liquid chromatography, voltammetry, combined gas chromatography–mass spectra, and by high performance liquid chromatography. Emphasis in this discussion is limited to the procedures used in the determination of phenol residues in honey, namely, the fluorometric technique, spectrophotometry utilising colorimetric methods, and the HPLC method.

2.4.2.1 Fluorometry

DiCesare and Ettre (1982) analysed phenol and aniline which are frequently found together in samples or in waste water. Both phenol and aniline were reported as closely eluting peaks on a column 100 mm X 4.6 mm I.D., consisting of a C_{18} bonded-phase. The mobile phase was acetonitrile-water (30: 70) at 2 mL/min. The excitation was set at 274 nm and emission at 325 nm. Separation of both phenol and aniline was achieved by adjusting the pH of the mobile phase prior to fluorescent detection. At low pH phenol is strongly fluorescent, whereas aniline is converted to a non-fluorescent anilinium cation. At high pH aniline is strongly fluorescent, whereas phenol is non-fluorescent as the phenoxide ion. Hence, by adjustment of the pH to enhance or suppress responses of fluorescing molecules, qualitation and quantitation can be optimized.

Fluorescence is an important analytical tool because of its sensitivity and specificity. Fluorometric methods can detect concentrations of substances as low as one part in ten billion, a sensitivity 1000 times greater than that of most spectrophotometric methods. The main reason for the increased sensitivity is due to direct measurement of the emitted radiation, which can be increased or decreased by adjusting the intensity of the exciting radiant energy. In fluorometric methods an increase in signal over a zero background is measured. The difference between the incident and the transmitted beams is measured indirectly in spectrophotometric methods. This results in a correspondingly large loss in sensitivity due to the small increase in the intensity of a very large signal which is measured in spectrophotometry.

The specificity of fluorescence is attributed to two factors. In fluorometry two wavelengths are used, excitation and emission. One factor contributing to the specificity is that two compounds which absorb radiation at one wavelength will probably not emit at the same wavelength. The second factor is that there are fewer fluorescent compounds than absorbing ones. All fluorescent compounds must absorb radiation, but not all compounds that absorb radiation fluoresce.

Fluorometry is used to determine compounds which possess native fluorescence, compounds which can be converted to fluorescent compounds (fluorophors) and those that quench the fluorescence of other compounds. Fluorescence is especially useful in trace analysis.

The disadvantage of fluorescence is that it is subject to interferences, such as the presence of impurities in low concentrations, temperature, pH, ionic strength, and presence of oxygen.

2.4.2.2 Spectrophotometry: Gibbs Method

Gibbs (1927) reported the identification of phenols by the blue indophenol color which resulted from the addition of 2,6-dichloroquinonechloroimide (2,6D).

Numerous workers have made detailed studies of the factors affecting the reaction of phenols with 2,6D. An investigation (Ettinger and Ruchhoft, 1948) revealed that the use of the Gibbs reaction followed by extraction of the resulting indophenol dye in n-butyl alcohol can result in an increase in sensitivity. The wavelengths for the maximum extinction of the reaction between phenol and 2,6D were listed as 670 nm in n-butyl alcohol and 610 - 630 nm in aqueous solution.

Colorimetric estimation of BHA in potato granules and in lard and shortening has been widely employed (Filipic and Ogg, 1960; Mahon and Chapman 1951a; 1951b;). In toxicological studies, the Gibbs reagent has been used for determination of phenol in the urine (Piotrowski,1971; Ohtsuji and Ikeda, 1972; Capel *et al*; 1972). Estimation of phosphatase activity in raw milk is conducted using 2,6D (Babel *et al*; 1978).

Colorimetric reactions, in general, are excellent for phenols. However, they are not completely specific. The reaction between phenol and the Gibbs reagent (2,6D) is shown in Figure 7. The reaction can occur in phenolic compounds with an ortho or para unsubstituted position (Dacre,1971). There are some exceptional cases in which highly substituted phenolic compounds with the para position unsubstituted do not give a positive test. Many compounds eg. amines (Castle,1950) and other amino derivatives (Fearon,1944) have been reported as

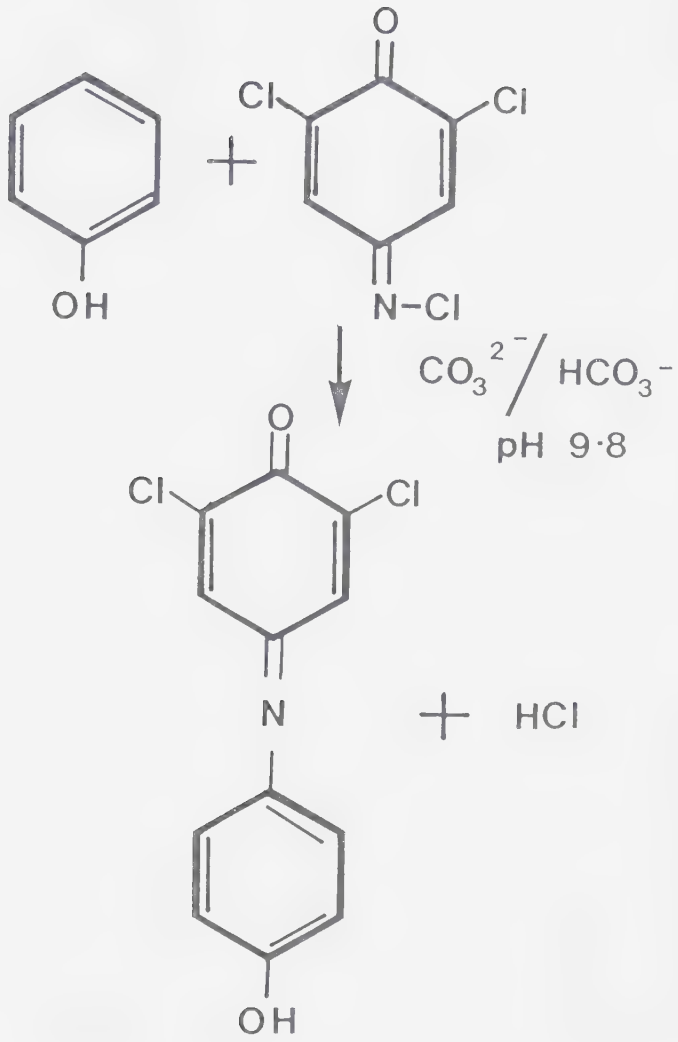


Figure 7. Equation showing coupling reaction of phenol with 2,6-dichloroquinonechloroimide to form an indophenol.

giving purple, violet or other characteristic colors.

2.4.2.3 Spectrophotometry: Aminoantipyrine Method

The reaction of phenols with 4-aminoantipyrine (4-AAP) was first described by Emerson (1943) and has been extensively applied in the determination of phenols in water.

In the Standard Methods for the Examination of Water and Waste Water, published by the American Public Health Association, the 4-AAP test is the procedure used for the determination of phenols. The colorimetric method involving the use of 4-AAP is employed in a Technicon CSM-6 AutoAnalyzer, which is used in water quality analysis (Marten and Zaleiko, 1969).

The reaction of phenol with the 4-AAP reagent in the presence of an oxidizing agent is shown in Figure 8. A concentrated ammonium hydroxide buffer at pH 10.2 was used. The dye produced can be extracted from aqueous solution with chloroform and the absorbance measured at 460 nm, or kept in aqueous solution and the absorbance monitored at 510 nm. The chloroform extraction method is reported to be capable of detecting 0.001 mg of phenols per litre of water, whereas the minimum detectable quantity by the direct photometric method is 0.1 mg phenols per litre of water.

The 4-AAP test has been applied to quantitate the phenols present in a number of pharmaceutical preparations (Johnson and Savidge, 1958).

In general, substances having a free phenolic hydroxyl group and a free para position or a para position substituted with halogen, hydroxyl or alkoxyl, sulphonic acid or carboxylic acid group give a positive reaction. The presence of a nitro or carboxylic acid group in the ortho position to the free hydroxyl group retards the the reaction (Johnson and Savidge, 1958; Emerson, 1943).

2.4.2.4 HPLC Determination

An extensive survey of the literature has shown the HPLC method to be the most popular for the determination of phenolic compounds. Determination of phenols and chlorophenols in dilute aqueous solutions has been performed on both normal and reverse phase columns (Brueggemann *et al.*, 1982; Armentrout

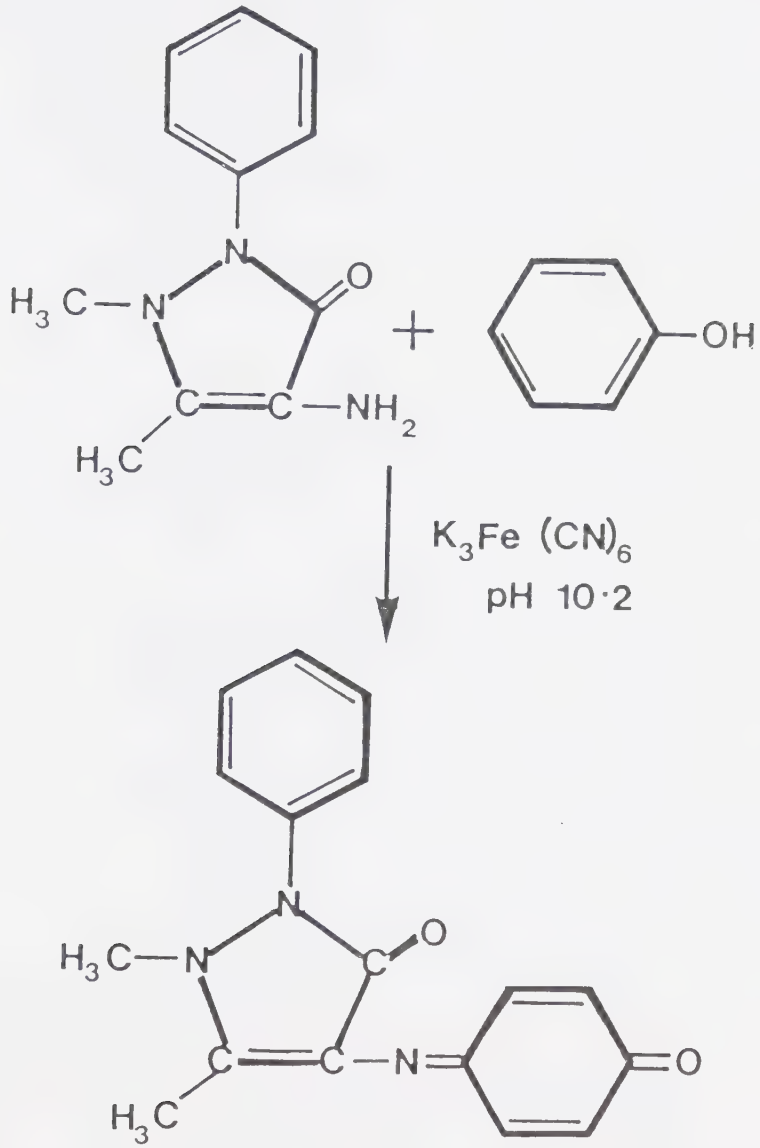


Figure 8. Equation showing coupling reaction of phenol with 4-amino-antipyrine in the presence of alkaline oxidizing conditions.

et al., 1979; Ivanov and Magee, 1973; Chao and Suatoni, 1982; Sporns, 1981).

Chao and Suatoni (1982) evaluated several HPLC reverse-phase columns and separated a total of thirty-eight phenolic compounds including phenol. Observation of the retention times shows a substantial difference between phenol and other phenolic substances.

In the majority of HPLC analyses detection of phenolic compounds incorporated ultraviolet detection. Electrochemical detection of phenolic compounds has also been utilized (Armentrout *et al.*, 1979). The separation of phenolic compounds by HPLC has been described in several reports using ion-exchange chromatography.

2.4.2.5 Evaluation of Methods

The HPLC method for detecting phenols is simple, rapid and accurate. There is no problem with the extraction of phenols from aqueous media and it is advantageous in that determination can be done directly without derivatization steps.

Gas liquid chromatography (GLC) has been used extensively for analysis of phenolic compounds, but most procedures require solvent extraction and subsequent sample concentration prior to analysis. Combined gas chromatography mass spectrometry (GC/MS) or selective derivatization and GC with electron capture detection have been reported (Lamparski and Nestrick, 1978). Disadvantages of GC methods are sample preparation time, cost of MS equipment, incomplete recoveries for most phenols, and the lack of detector selectivity (Armentrout *et al.*, 1979).

Identification of isolated phenols from plants was done using reverse phase thin layer chromatography on a Whatman KC_{18} F reversed phase layer (Sherma and Sleckman, 1981). However, accurate quantitation is not as easily carried out as in other methods.

2.5 Sensory Evaluation

Sensory evaluation is directly concerned with human evaluation and measurement of physical stimuli. Factors such as testing environment, sample preparation and method of presentation are controlled in order to minimize external influence on judgment. Another important consideration is the selection and training of panelists.

Sensory evaluation panels can be grouped into three types: (a) highly trained experts, (b) laboratory panels, and (c) large consumer panels.

The trained experts evaluate quality and can be of particular value in the assessment of product changes for which there is no adequate instrumentation. Evaluations by experts and trained laboratory panels are useful in improving quality control and flavor formulations. Consumer panels are useful in predicting acceptability of marketable products.

There are fundamentally three types of sensory tests. They are preference/acceptance, discriminatory and descriptive tests. Preference and acceptance tests are subjective to the panelists' response. Discriminatory tests (triangle, paired comparison, duo–trio) are used to determine whether differences exist between products. Descriptive tests are used to determine the nature and intensity of differences and include threshold tests. These tests are classified and documented (Larmond, 1977). Description will be limited to the triangle test and the Signal Detection Test (O'Mahony, 1979). In the triangle test the panelist is presented with three coded samples and is informed that two are the same and one is different. He is requested to identify the odd sample. The triangle test is often used as a preliminary test in the selection of panelists. Analysis of the results of the triangle test is based on the probability that if there is no detectable difference the odd sample is selected by chance one third of the time. Tables for determining significance in the triangle tests have been prepared (Roessler *et al.*, 1978). The Signal Detection Test is a short–cut method of measuring multiple differences. It has been used for testing off–flavors in milk (O'Mahony, 1979) and the differences are measured either by ranking or rating.

In the Signal Detection Test panelists are presented with a number of samples and are asked to compare each sample with a control provided. The judges are then required to indicate whether there is a difference and whether they are sure of their judgment. From the data the probability, an R-index is calculated. The R-index is the probability of obtaining a correct identification. The data is calculated by a method developed by Brown (1974). The literature (ASTM, 1978) gives several threshold values of phenol in different products (Table 1). It can be seen that the threshold values for both beer and synthetic deodorized butter are lower. This is expected since highly trained panelists were chosen for the assessment of flavor. For the flavor threshold of phenol in synthetic butter (Urbach *et al.*, 1972) the samples were presented in a series of increasing concentration beginning with a control (no phenol). The difference in concentration between samples was usually by a factor of 10, although in some cases it was lower. At values of 20 ppm panelists reported a strong carbolic taste. It is of interest to note that a laboratory panel found the flavor threshold for phenol in synthetic deodorized butter at least 100 times greater than the value determined by the professional graders.

TABLE 1

Literature Values[1] of Threshold Determinations of Phenol

Product	Modality	Threshold Type	Threshold Value (ppm)
water	taste	detection	25
water	odor	detection	5.9
synthetic deodorized butter	taste	detection	0.01
beer	taste	recognition	0.0271
beer	taste	detection	0.0125

[1]ASTM Committee E-18. 1978. Compilation of Odor and Taste Threshold Values Data.

3. EXPERIMENTAL

3.1 HPLC Equipment and Conditions for HPLC Separations

The HPLC system consisted of a Beckman Model 110A pump (Beckman Inc., Fullerton, CA) controlled by a Model 420 Microprocessor Controller (Altex Scientific, Inc., Berkeley, CA). A Whatman Solvecon column, 250 mm x 4.6 mm ID packed with 37-53 μm silica gel was placed before the injector. The HPLC separation on a normal phase column was similar to the method described by Sporns (1981) and was performed using a Partisil PXS 5/25 column, (Whatman Inc., Clifton, NJ) protected by a Whatman HC Pellosil guard column. A Partisil ODS-3 RAC column, 100 mm x 9.4 mm ID (Whatman) fitted with a Whatman Co: Pell ODS Guard column, was employed for reverse phase separation, according to a modification of the procedure described by Chao and Suatoni (1982). The solvent system for normal phase separation consisted of water from a Millipore Milli-Q system (Millipore Corp., Bedford, MA) which was degassed prior to use and maintained at a flow rate of 1 mL/min. The eluent for the reverse phase separation was acetonitrile (HPLC grade): water (1:1, v/v) maintained at a flow rate of 2 mL/min. Analyses were carried out on both columns at ambient temperature. Samples were injected using a 20 μL Alltech injector (Alltech Associates, Inc., Deerfield, 1L) and the absorbance was monitored by a Spectro Monitor III Model 1204A detector (Laboratory Data Control, Riviera Beach, FL) set at 195 nm. Absorbance was also monitored at different wavelengths, but mainly at 195 nm. Chromatographic data was recorded using a Hewlett-Packard integrator (Hewlett-Packard, Route 41, Avondale, PA) or a model 355 Cole Parmer recorder (Cole Parmer, Irvine, CA).

3.2 Preparation of Standard Solutions

All water was purified using a Millipore Milli-Q system. Solutions were prepared in all glass apparatus fitted with glass stoppers and were prepared daily, except where noted.

Concentrated phenol solution: 1 g of phenol crystal was weighed to 1 mg and diluted to 1 L with water. The solution was protected from light and kept for a period not exceeding 1 month.

Dilute phenol solution: 5 mL of concentrated phenol solution were diluted to 500 mL with water.

Concentrated 2,6-dimethylphenol solution: 0.4 g of 2,6-dimethylphenol, recrystallized from hexane, was weighed to 1 mg and diluted to 1 L with water. The solution was protected from light, refrigerated and kept for a period not exceeding 4 days.

Dilute 2,6-dimethylphenol solution: 5 mL of concentrated 2,6-dimethylphenol were diluted to 200 mL with water.

Mixed standard solution: 5 mL of dilute phenol solution and 10 mL of dilute 2,6-dimethylphenol solution were combined and diluted to 50 mL with water.

Concentrated 4-chlorophenol solution: 1 g of 4-chlorophenol crystals was weighed to 1 mg and diluted to 1 L with water. The solution was protected from light and kept for a period not exceeding 1 month.

Dilute 4-chlorophenol solution: 10 mL of concentrated 4-chlorophenol solution were made up to 250 mL with water.

Mixed standard solution: 4 mL of dilute 4-chlorophenol solution and 5 mL of dilute phenol solution were combined and diluted to 50 mL with water.

3.3 Sample Preparation

A 10 g sample of honey or wax was weighed and transferred into an 800 mL Kjeldahl flask with the aid of 30 mL of water. For the HPLC procedure (used only for the analysis of honey), 25 mL of water and 5 mL of aqueous internal standard solution were added. The mixture was steam distilled using a Büchi 320 Nitrogen Distillation Unit (Büchi 320, Foss Electric Canada Ltd., Cornwall, Ontario) and the first 10 mL fraction of the steam distillate was collected. Precautions were taken to prevent cross-contamination between samples. The Kjeldahl apparatus was cleaned by steam distilling from another

Kjeldahl flask until 100 mL of steam distillate were collected. Steam distillation of wax samples resulted in "waxy" deposits which frequently required repeated steam distillations between samples to clean the Kjeldahl unit.

3.3.1 HPLC Analysis of Honey

A 20 µL sample of the steam distillate was injected immediately into the liquid chromatograph and phenol and the internal standard were detected at 195 nm. Injections of the samples were alternated with injections of the mixed standard, and measurements of the peak heights were recorded from the chromatograms.

3.3.1.1 Identification and Quantitation from HPLC Chromatograms

Quantitation of phenol required a factor (correction factor) to account for the difference in volatility between phenol and the internal standard. The methodology of standard addition of known concentrations of phenol solution to honey (free of detectable phenol) was employed. The procedures described in Sections 3.3 and 3.3.1 were followed. Using regression analysis, the correction factor was computed by plotting a graph of phenol found against phenol added. The following equation summarizes the quantitation of phenol content (ppm) by peak height (pk. ht.) comparison with the mixed standard (std.) and the sample (sam.).

Phenol (ppm) = [pk. ht. phenol (sam.)] / [pk. ht. internal standard (sam.)]
x [pk. ht. internal standard (std.)] / [pk. ht. phenol (std.)]
x [µg phenol (std.)] / [µg internal standard (std)]
x [µg internal standard (sam.)] / [wt. honey (g.)]
x correction factor.

3.3.2 The Gibbs Method of Analysis of Honey and Beeswax

3.3.2.1 Reagents

Gibbs reagent (0.5%, w/v): A concentrated solution was prepared by dissolving 0.5 g of 2,6-dichloroquinonechloroimide (CQC) and diluting to 100 mL with 95% ethanol. The solution was protected from light, refrigerated and kept for a period not more than 1 week.

Gibbs reagent (0.04%, w/v): 4 mL of the above reagent were removed and diluted to 50 mL with 95% ethanol. The solution was protected from light and prepared fresh each day.

Buffer: A pH 9.8 buffer was prepared by dissolving 60 g sodium carbonate monohydrate and 40 g sodium bicarbonate in 1 L of water.

3.3.2.2 Procedure

To the 10 mL of steam distillate obtained as in Section 3.3, 1 mL of buffer was added, followed by 1 mL of 0.04% Gibbs reagent (Gibbs, 1927). After each addition, the mixture was thoroughly shaken to ensure complete mixing. The flask was allowed to stand for 5 min and then placed for 30 min in a water bath maintained at 40°C. The absorbance of the solution was measured at once using a Model DU 8 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) set at 610 nm. All absorbance measurements were made against a reference blank containing 10 mL water, 1 mL buffer and 1 mL Gibbs reagent.

3.3.2.3 Determination of Phenol Levels in Honey and Beeswax by the Gibbs Method

The procedure was followed as in Section 3.3.2.2 and the absorbance of the sample was measured. The phenol content was quantitated by extrapolating from a phenol standard calibration and multiplying by a correction factor determined from regression analysis. The correction factor was established by standard addition of known concentrations of phenol to both honey and beeswax (free of phenol).

3.3.3 The Aminoantipyrine Method of Analysis of Honey and Beeswax

3.3.3.1 Reagents

Aminoantipyrine solution: 2.0 g of 4-aminoantipyrine were weighed and diluted to 100 mL with water. The solution was prepared fresh each day.

Potassium ferricyanide: 8.0 g of potassium ferricyanide were weighed and diluted to 100 mL with water. The solution was prepared fresh each day.

Ammonium chloride solution: 50 g ammonium chloride were dissolved and diluted to 1 L. The pH of the solution was adjusted to 10.2 with concentrated ammonium hydroxide.

3.3.3.2 Procedure

To the 10 mL of steam distillate obtained as in Section 3.3 were added 1 mL of the ammonium chloride solution, followed by 1 mL aminoantipyrine and 1 mL of potassium ferricyanide. After each addition, the flask was shaken to ensure complete mixing. The absorbance was read at 510 nm on a spectrophotometer and the reading from a blank prepared using 10 mL of water in place of the steam distillate was subtracted.

3.3.3.3 Determination of Phenol Levels in Honey and Beeswax by the Aminoantipyrine Method

A calibration curve for phenol was plotted and a correction factor determined similarly as in Section 3.3.5.2. Assay of phenol was analogous to the Gibbs method.

3.3.4 Fluorometric Method of Analysis of Honey and Beeswax

3.3.4.1 Procedure

The 10 mL of steam distillate obtained as in Section 3.3. was measured directly using a Perkin–Elmer 650–10LC fluorescence spectrophotometer, (Perkin–Elmer, Norwalk, Connecticut, U.S.A.) attached to a Perkin–Elmer Model 150 Xenon Power Supply. The excitation wavelength and emission wavelength were monitored at 270 nm and 300 nm respectively, with both excitation and emission slit widths set at 5 nm. The fluorescent intensity was measured on a Varian A–25 recorder. The recorder deflection due to phenol in the sample was measured and the deflection from a water blank was subtracted.

3.4 Field Trip

3.4.1 Application of Phenol

The field trials, using phenol, were conducted at several locations near Edmonton, Alberta, Canada, with the co-operation of a local beekeeper.

Phenol crystals (F.W.Jones and Son Ltd., Beekeepers' Supplies, Matériel Apicole, Bedford, Quebec) were dissolved in water to obtain a 90% (w/v) phenol solution. This concentrated phenol solution (10 mL) was applied to the adsorbent cloth on the underside of the phenol board. Phenol boards were supplied by the beekeeper and had been unused for at least one year. After application of the concentrated phenol solution, the phenol board was immediately placed on top of the hive. The time from application of the phenol solution to placing the phenol board on the hive was about four minutes. The surface temperature was recorded using a temperature probe fastened to the upper surface of the phenol board. Sufficient time was allowed for the repulsion of the bees from the top super before the phenol board was removed. The upper super was then removed for sampling and the phenol board placed on top of the next super. The time of exposure of the honey super to the phenol board was recorded. The process involving the consecutive removal of the uppermost super and placing of the phenol board on the next lower honey super was repeated until all the honey supers were collected and only two supers remained (brood supers). Plate 7 illustrates the hive before collection and Plate 8 the hive after application of phenol.

3.4.2 Collection of Samples

Frames were chosen from the removed supers and sampled as soon as possible by first marking an area with the circular top of a glass collection jar (5.4 cm in diameter). A number of impressions were made on the frame so that a sufficient quantity of wax and honey samples was collected for analysis. The location of the frame in the super and the average distance from the top of the frame to the middle of the impressions was recorded. Samples were

Plate 7

Phenol boards placed on top of hives prior to collection of honey from honey supers

Plate 8

Clustering and displacement of bees near end of honey collection after application of phenol

removed from capped and uncapped honey cells (Plate 9). Samples were also collected after scraping the surface wax from capped cells. All honey and wax samples collected from the impressions were placed in collection jars with the aid of stainless steel spoons and sealed for later analysis in the laboratory.

Samples of honey and wax were collected prior to exposure to the phenol board. Also, several samples of wax and honey were obtained from different beekeepers.

3.4.3 Sample Analysis

In the laboratory the samples collected were weighed and divided into two fractions. One fraction was stored at ambient temperature in the sealed collection jar for later analysis (one month or more) and honey from the other fraction was separated from wax using a cotton cheesecloth. Honey, separated from the wax, was immediately analysed and the remainder was left at ambient temperature in an unsealed jar (exposed to air) for analysis at a later date. Duplicate analyses of each sample were carried out whenever possible, depending on the sample size.

Analytical methods for phenol residues in wax and honey are described in Section 2.4.

3.5 Sensory Evaluation

3.5.1 Procedure

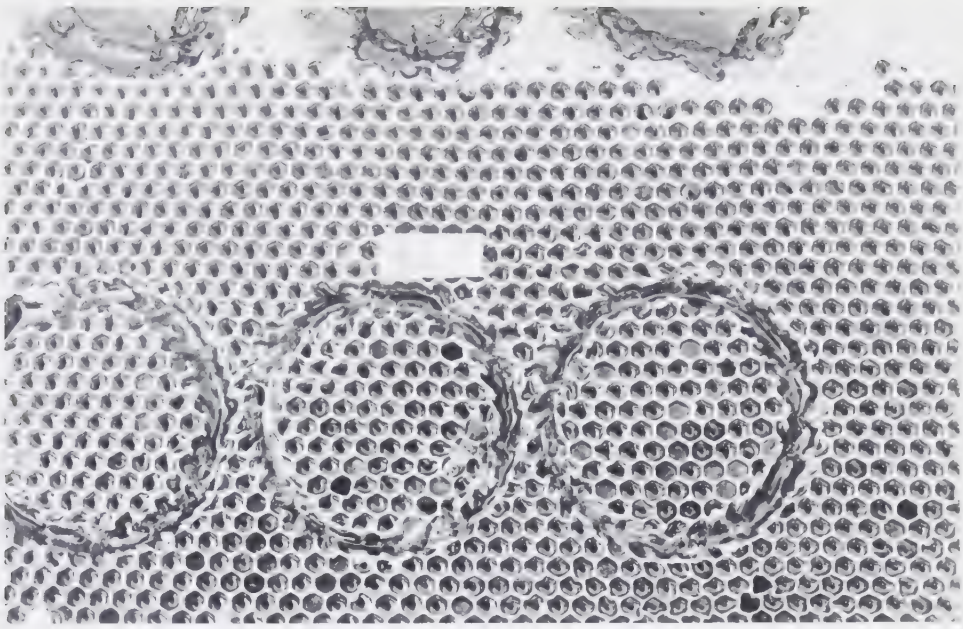
Threshold determinations of phenol in honey and in water were performed using the Signal Detection Test (O'Mahony, 1979).

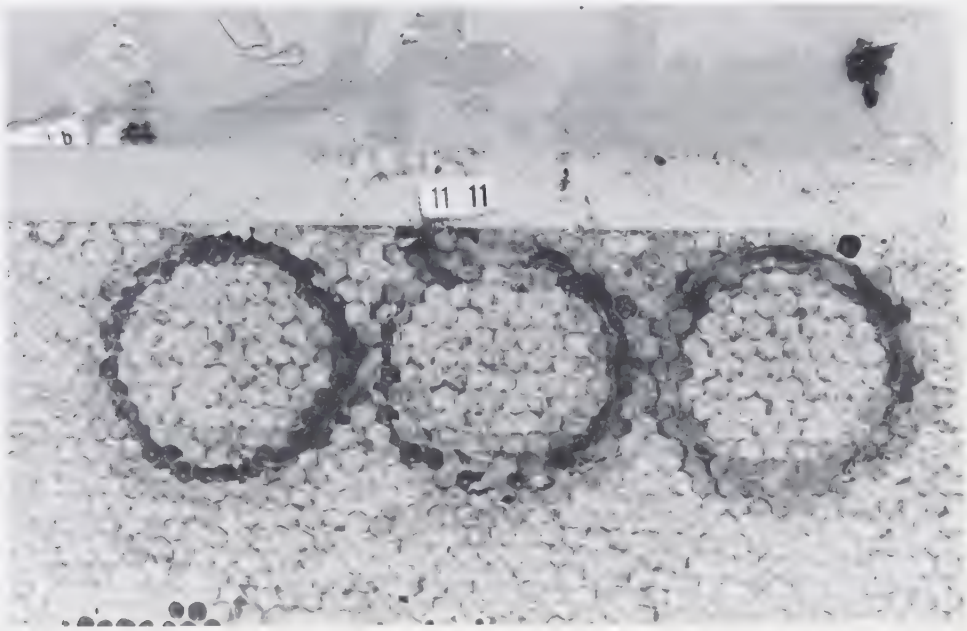
Preliminary investigations were conducted using the triangle test (Larmond, 1977) to measure differences in samples of honey which varied in the amount of phenol added. In the triangle test, (Appendix 1), panelists were required to choose the odd sample and indicate the degree of difference between the duplicates and the odd sample.

Plate 9

Uncapped honey cells observed in circular impressions (Top)

Capped honey cells observed in circular impressions (Bottom)

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Testing was done periodically over a year from Monday to Friday with sessions held from 10 A.M–12 NOON in the morning or from 1.30 P.M.–2.30 P.M. in a taste panel room equipped with individual booths.

The panel comprised 10–20 members of the staff and students of the Department of Food Science at the University of Alberta, and was untrained. The members, both men and women, were randomly selected and participated throughout the period of sensory evaluation. The composition of the panel varied slightly during the course of the tests as some members were unavailable from time to time.

In the Signal Detection Test (Appendix 2) a reference, labelled R, either distilled water or honey (no detectable phenol), was included. The reference was not identified to the panelists. Samples, either honey or water to which phenol was added, were presented to the panelists in an ascending order of concentration. It should be noted in each case, at least one of the samples coded with a three digit number included an internal reference (no phenol).

The threshold determination of phenol in water was conducted under white incandescent lighting in the taste panel room. Red illumination was provided for the threshold determination of phenol in honey to mask any visual difference between the samples.

The appropriate concentrations of phenol for spiking were achieved by dissolving phenol crystals, purified by sublimation, in distilled water. A Hamilton microliter syringe (Hamilton Co., Reno Nevada) was employed for the addition of phenol to the honey or water.

The honey used throughout the sensory evaluation was supplied from a beekeeper in Alberta, who collected honey without the use of a phenol board. Analysis by HPLC confirmed that the honey was free of detectable phenol. The honey stored at 4°C was liquefied by immersing in a glass jar on a water bath set at 47°C. After an incubation period of 3 h and 28 min the warm liquefied honey was removed and spiked using the phenol solution prepared as in above. The glass jar was immediately sealed. A magnetic stir bar placed in the jar before sealing ensured that the process of mixing on a magnetic plate for 15

min was uniform.

Preparation of spiked honey samples commenced two days prior to the taste panel sessions. Confirmation of the phenol level was ascertained by HPLC analysis one day prior to the serving of the samples.

Each honey sample, averaging 10 mL was placed in a glass test tube (125 mm X 16 mm) which was provided with a glass rod (170 mm X 6 mm) for testing. The samples were served in a test tube rack. Instructions were given to the panelists to consume a minimum of one unsalted soda cracker and rinse the mouth with distilled water in between samples. Samples could be swallowed or expectorated into a sink in each tasting booth.

The determination of the taste threshold of phenol in water was conducted in one session using the Signal Detection Test. The phenol solutions were prepared as outlined previously, 12 h before the panel session and were served in glasses. Panelists were instructed to taste a small volume of the solution slowly and hold in the mouth for a few seconds before expectorating. Distilled water was provided for rinsing the mouth in between samples. Distilled water was used as the reference, for preparation of all solutions, and for rinsing of the mouth instead of Milli-Q water after a number of volunteers assessed the taste to be more acceptable. The distilled water was analysed by HPLC and found to be free of detectable phenol.

All apparatus (test tubes, rods, glasses) was thoroughly washed in hot soapy water and rinsed several times (last time with distilled water) before each tasting session to prevent any carry-over effects.

4. RESULTS AND DISCUSSION

4.1 Methods of Analysis of Phenol in Honey and Beeswax

4.1.1 High Performance Liquid Chromatography (HPLC)

The use of reverse phase HPLC conditions, similar to the procedure of Chao and Suatoni (1981) gave excellent separation of phenol from other substituted phenolic compounds and permitted repeated analyses of phenol with high resolution and unimpaired column efficiency.

Figures 9 and 10 illustrate two typical chromatograms obtained on the HPLC reverse phase column. It can be seen that the peaks are well separated. The two peaks were identified by comparing the retention times with that of the mixed standard injected under the same conditions. The first peak had the same retention time as phenol and the second corresponded to the appropriate internal standard.

For normal phase HPLC conditions, the change from a Spectral Physics Spherisorb 5 μ silica column (Sporns, 1981) to a Whatman Partisil PXS 5/25 analytical column resulted in phenol being almost unretained. Additional problems observed were peak broadening of the standards, split peaks, and with time there was a gradual deterioration of the column, which could not be regenerated according to the solvent sequence suggested by the manufacturer.

The variation from normal phase conditions to reverse phase conditions required the use of a solvent of the following composition 50:50, acetonitrile: water. The use of acetonitrile contributes to the expense of the analysis.

The internal standard method on which quantitation of phenol is based provides an accurate determination, since any loss of phenol is reflected in a corresponding loss in the internal standard. Both internal standards, 2,6-dimethylphenol and especially, 4-chlorophenol were highly suitable since the retention times were similar to phenol, but yet allowed an excellent separation. Both internal standards are crystalline solids, although 2,6-dimethylphenol is not as stable in solution as 4-chlorophenol. Spectrophotometric scans of the internal

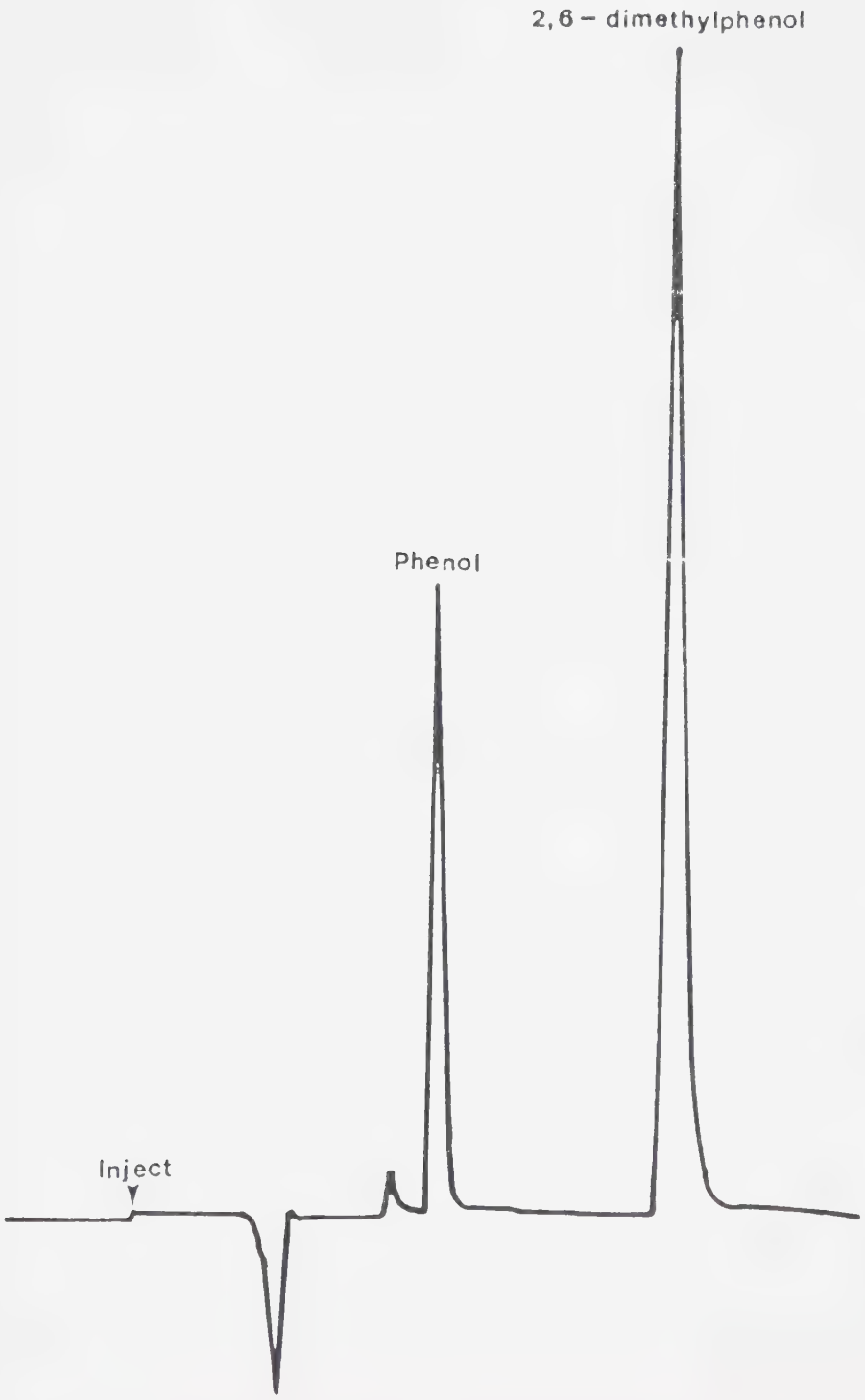


Figure 9. HPLC chromatogram of the steam distillate of a honey containing 5.7 ppm phenol using 2,6-dimethylphenol as the internal standard and monitored at 195 nm on the reverse phase column.

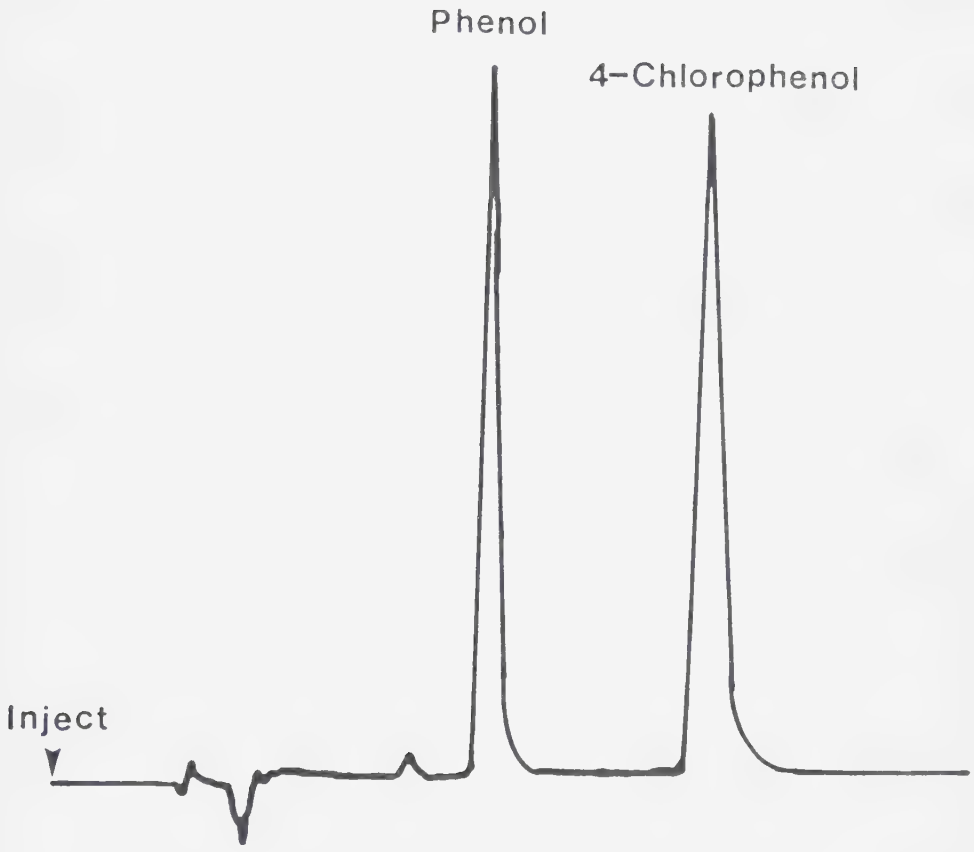


Figure 10. HPLC chromatogram of the steam distillate of a honey containing 7.6 ppm phenol using 4-chlorophenol as the internal standard and monitored at 195 nm on the reverse phase column.

standards were examined to ensure that no deterioration had occurred.

The chosen method for analysis of phenol is the HPLC method using reverse phase conditions and 4-chlorophenol as the internal standard.

In the HPLC method variations with respect to changes in the amounts or volumes used in the steam distillation would alter the correction factor. However, slight variations would have a negligible effect. Appropriate dilution is necessary for samples of honey and wax which contain very high levels of phenol.

Unfortunately, the determination of phenol in wax could not be adapted to analysis by the HPLC procedure. The major problem arising was the steam distillation of components from the wax, which interfered with the detection of phenol. Also, some of the compounds had very long retention times for the solvent conditions causing increased problems after repeated injections.

4.1.1.1 Quantitation of Phenol by HPLC Method

The quantitation of phenol involved the use of the equation for the internal standard method based on peak height measurements as described by Sporns (1981) and summarized in Section 3.3.1.1. Calculation of phenol using the internal standard, 2,6-dimethylphenol or 4-chlorophenol, required the multiplication of the equation by a factor which accounted for the difference in volatility of phenol and the internal standard. The order of the volatility of the compounds is as shown, 2,6-dimethylphenol > phenol > 4-chlorophenol. The factors were determined by adding phenol to honey (free of phenol) at levels ranging from 0.5 to 30 ppm in the presence of the internal standard, and quantitating the phenol recovered. The results were linear over the range tested and are presented in Tables 2 and 3. The linear regression equations relating phenol calculated (y) vs phenol actually added (x) are given in Table 4.

TABLE 2

Recovery of Phenol from Spiked Honey by HPLC using the Internal Standard, 2,6-dimethylphenol.

Phenol Added (ppm)	Phenol Calculated (ppm)
0.503	0.21
1.06	0.36
5.03	1.42
5.08	1.52
8.05	2.33
10.06	3.16
10.15	3.28
13.08	3.70
15.09	4.59
15.23	4.76
20.12	5.82
25.20	7.12
25.37	7.42
27.16	9.08

TABLE 3

Recovery of Phenol from Spiked Honey by HPLC using the Internal Standard, 4-chlorophenol.

Phenol Added (ppm)	Phenol Calculated (ppm)
1.00	2.10
5.02	8.63
8.03	13.70
10.04	16.46
15.06	24.82
15.06	25.00
20.08	34.35
25.00	35.30
30.00	49.90

TABLE 4

Linear Regression Equations Showing Relationship between Phenol Calculated (y) and Phenol Actually Added (x) for the HPLC Method.

Equation	Standard Regression Coefficient	Internal Standard	Correction[1] Factors
y = 0.30258 x	0.998	D	3.305
y = 1.60890 x	0.998	4-C	0.6215

[1] calculated as inverse of slope (1/m).

D = 2,6-dimethylphenol

4-C = 4-chlorophenol

4.1.2 Colorimetric Determinations

4.1.2.1 Colorimetric Determination with the Gibbs Reagent

Several workers (Ettinger and Ruchhoft, 1948; Mahon and Chapman, 1951b) have reported that the reaction between phenol and the Gibbs reagent is highly dependent on factors such as pH and temperature. The effect of pH studied over the range of 9.2 to 9.8 using a series of borate and sodium bicarbonate/carbonate buffers demonstrated that maximum color formation occurred at pH 9.8 using the bicarbonate/carbonate buffer. A spectrophotometric scan (Figure 11) showed maximum absorption at a wavelength of 610 nm. A study of the effect of the concentration of the Gibbs reagent was done using 0.01 to 3% CQC in 95% ethanol. A 0.04% Gibbs reagent was determined to be suitable for development of the blue indophenol color at 610 nm. Higher concentration resulted in a significant absorbance in the blank. The color stability monitored after incubation of samples in a water bath at 40°C for 30 min under the conditions stated above, showed the color to be stable at least for 1 h. The 0.04% Gibbs reagent prepared from a dilution of the stock solution, which was refrigerated, gave a consistent reading for each set of determinations when read against butanol.

4.1.2.2 Colorimetric Determination with the Aminoantipyrine Reagent

A spectrophotometric scan of a honey sample indicated that the wavelength of 510 nm was suitable for absorbance readings (Figure 12). There is a high end absorption in the region of 450–475 nm. The end absorption, however, does not interfere in the determination and appears to be contributed by the absorbance of the reagents in the blank and in the sample. It is important to read the absorbance of the samples immediately since the color stability was observed to decrease within 15 min.

Figure 11. Spectrophotometric scan of honey distillate between 225-700 nm using the Gibbs method and read against a reagent blank.

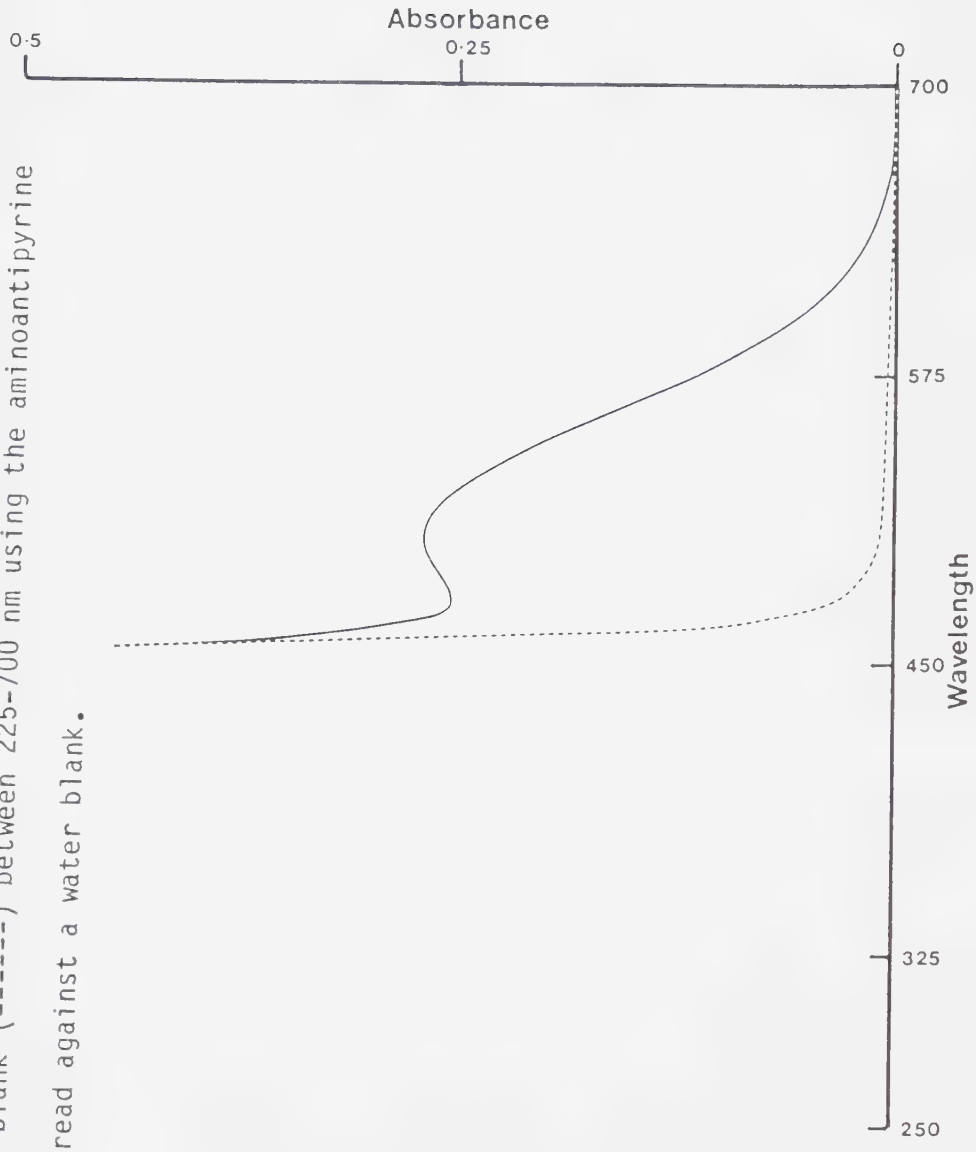


Figure 12. Spectrophotometric scans of honey distillate (———) and reagent blank (------) between 225-700 nm using the aminoantipyrine method and read against a water blank.

4.1.3 Fluorometric Method

The separation of phenol from aniline (DiCesare and Ettre, 1982) was performed by the fluorescent method.

Initial scanning of a solution of phenol was conducted to determine suitable excitation and emission wavelengths. The greatest fluorescent intensity was observed at 300 nm at an excitation wavelength set at 270 nm. The excitation and emission slit widths were both set at 5 nm.

Notably, a fluorometric investigation of phenol in honey dissolved in water revealed a significantly high interference from the honey thereby, discounting the possibility of a direct determination of phenol in honey. Hence, isolation of phenol by steam distillation of the honey was determined to be a preliminary step for the subsequent analysis of honey.

Emission and excitation scans of the steam distillates (Figures 13 and 14) from samples of a clear and dark honey (free of detectable phenol) revealed a negligible background absorbance at an exitation wavelength of 270 nm and an emission wavelength of 300 nm. Thus, the selected emission and excitation wavelengths were confirmed to be appropriate for analysis of phenol in honey samples. A scan of the water used as a blank showed the water to be free of interfering substances.

4.1.4 Quantitation of Phenol by the Gibbs Method, the Aminoantipyrine Method and the Fluorometric Determination.

Method and the Fluorometric Determination.

Calibration curves (Figures 15–17) for each method were obtained using standard phenol solutions. Regression analysis of the data from the calibration curves yielded the relations between phenol actually added (x) and phenol calculated (y) for the Gibbs, the aminoantipyrine, and fluorometric methods (Table 5). From the table, the correction factors for each method are computed by obtaining the inverse of the slope from each equation. These correction factors relate absorbances or recorder deflection measured to the concentration of phenol. It should be noted that the calibration curve (Figure 17) for the

Figure 13. Scans of emission of phenol solution (5 ppm), and distillates of both clear and dark honey containing zero phenol by fluorometry, at excitation wavelength set at 270 nm.

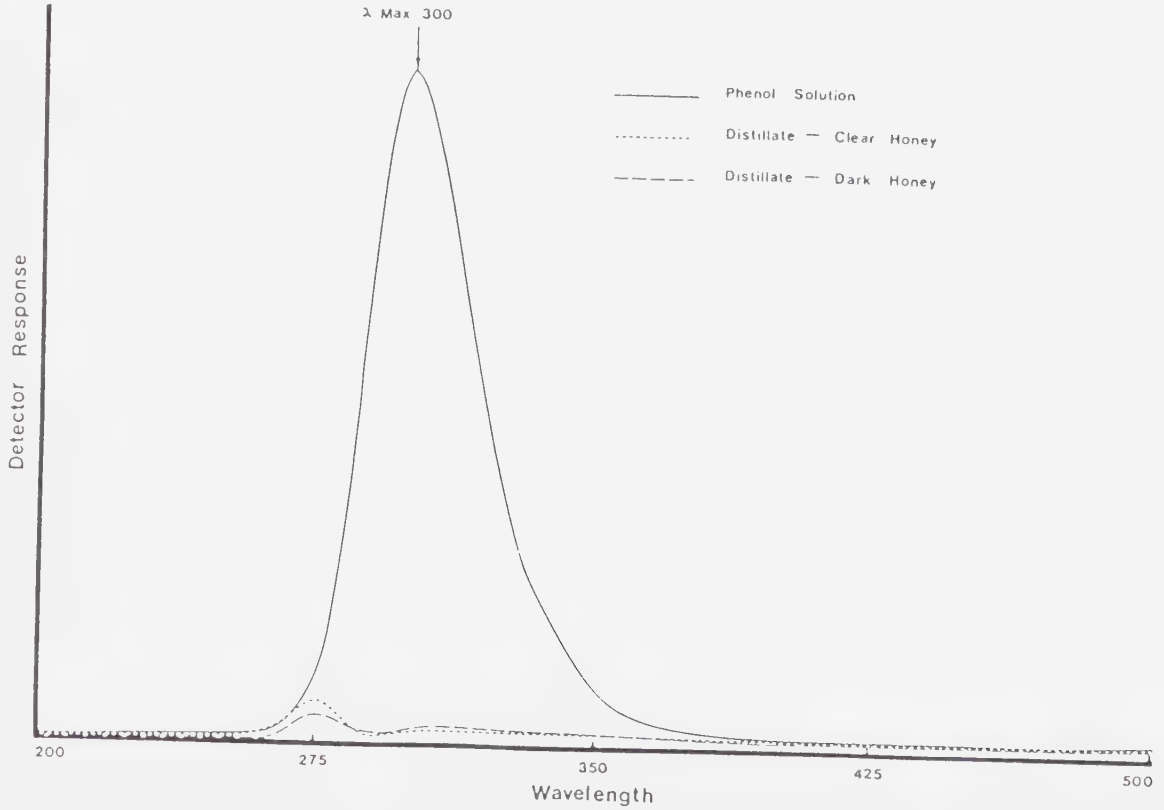
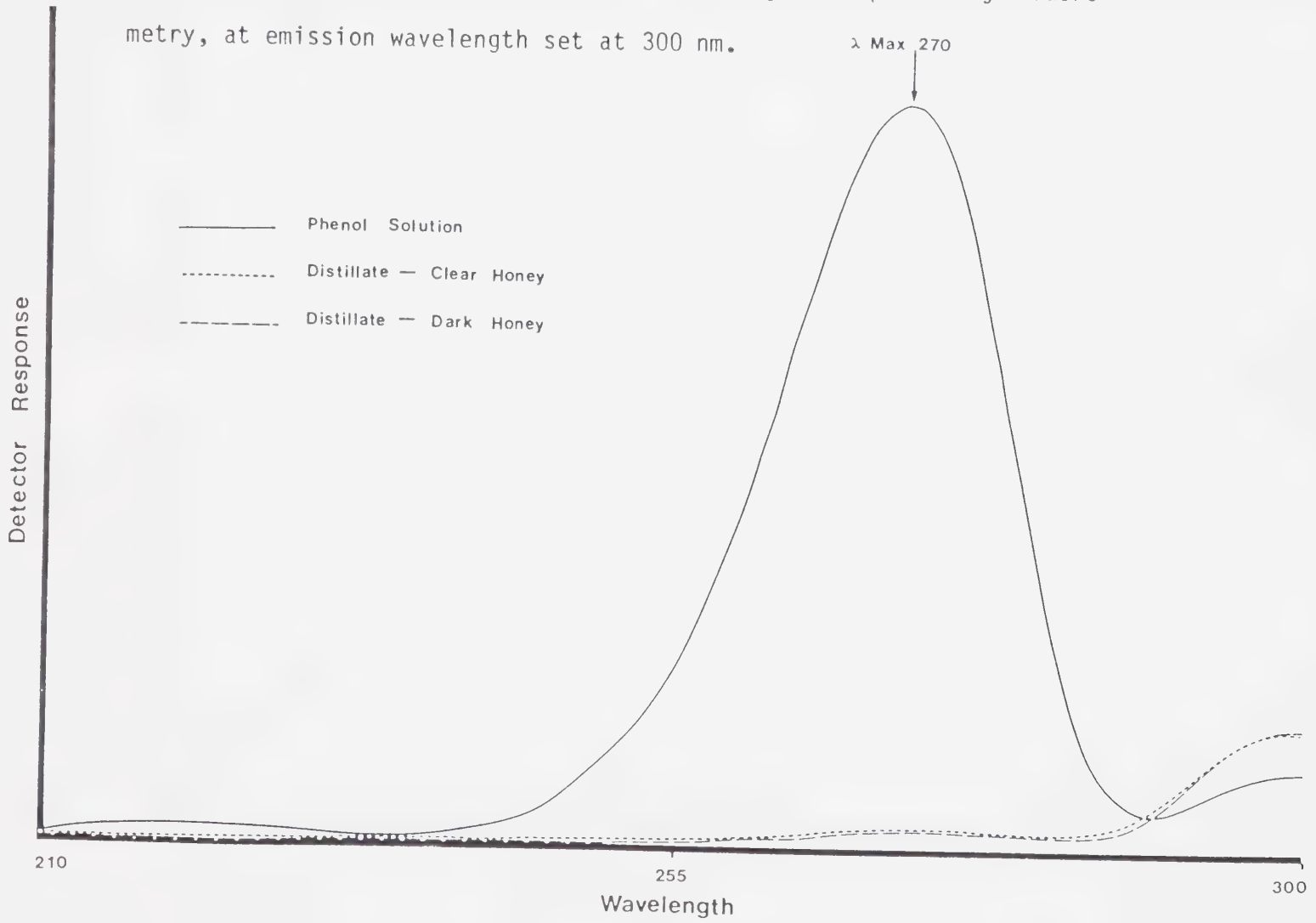


Figure 14. Scans of excitation of phenol solution (5 ppm), and distillates of both clear and dark honey containing zero phenol by fluorometry, at emission wavelength set at 300 nm.



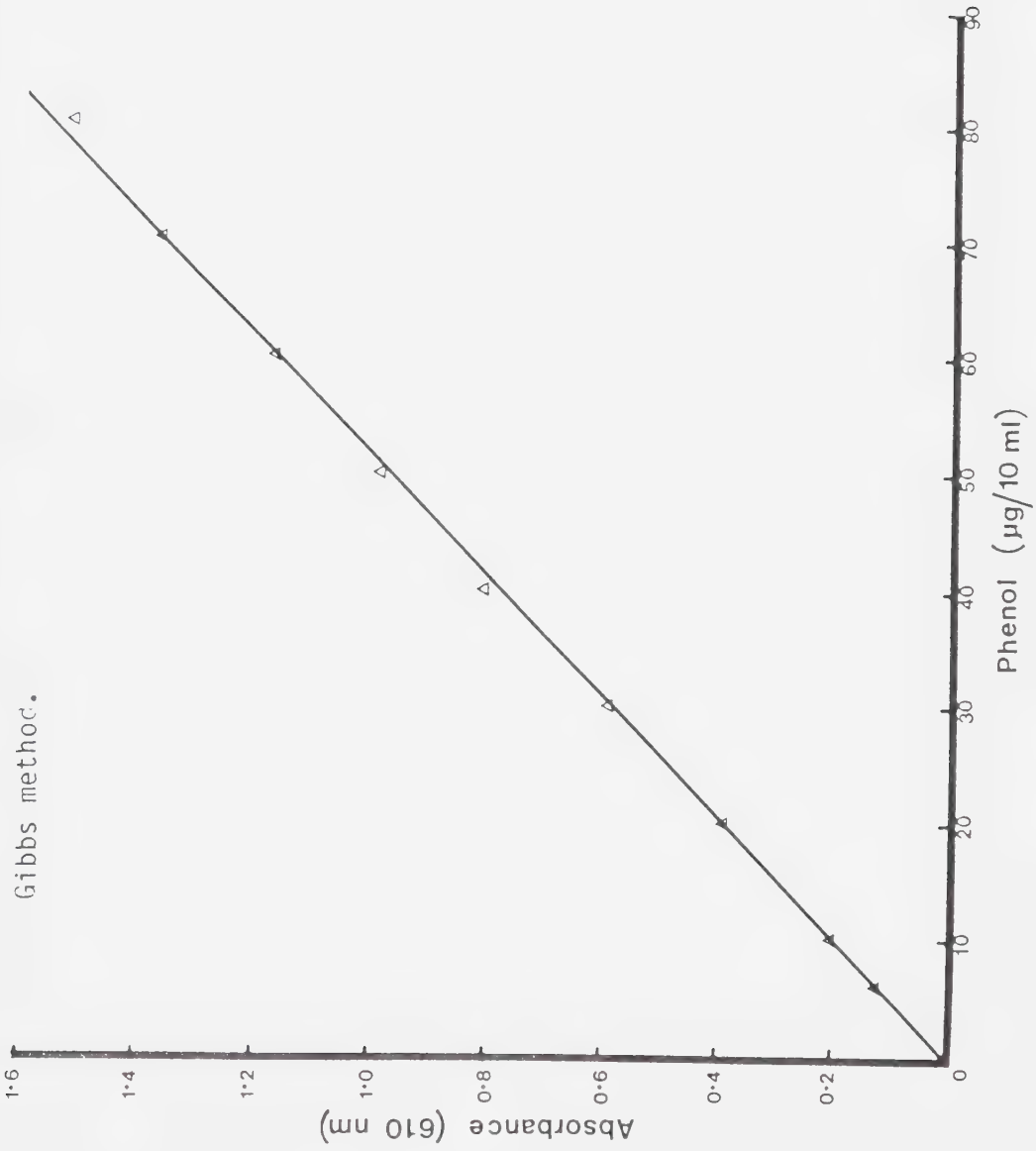


Figure 15. Calibration curve of standard phenol solution using the Gibbs method.

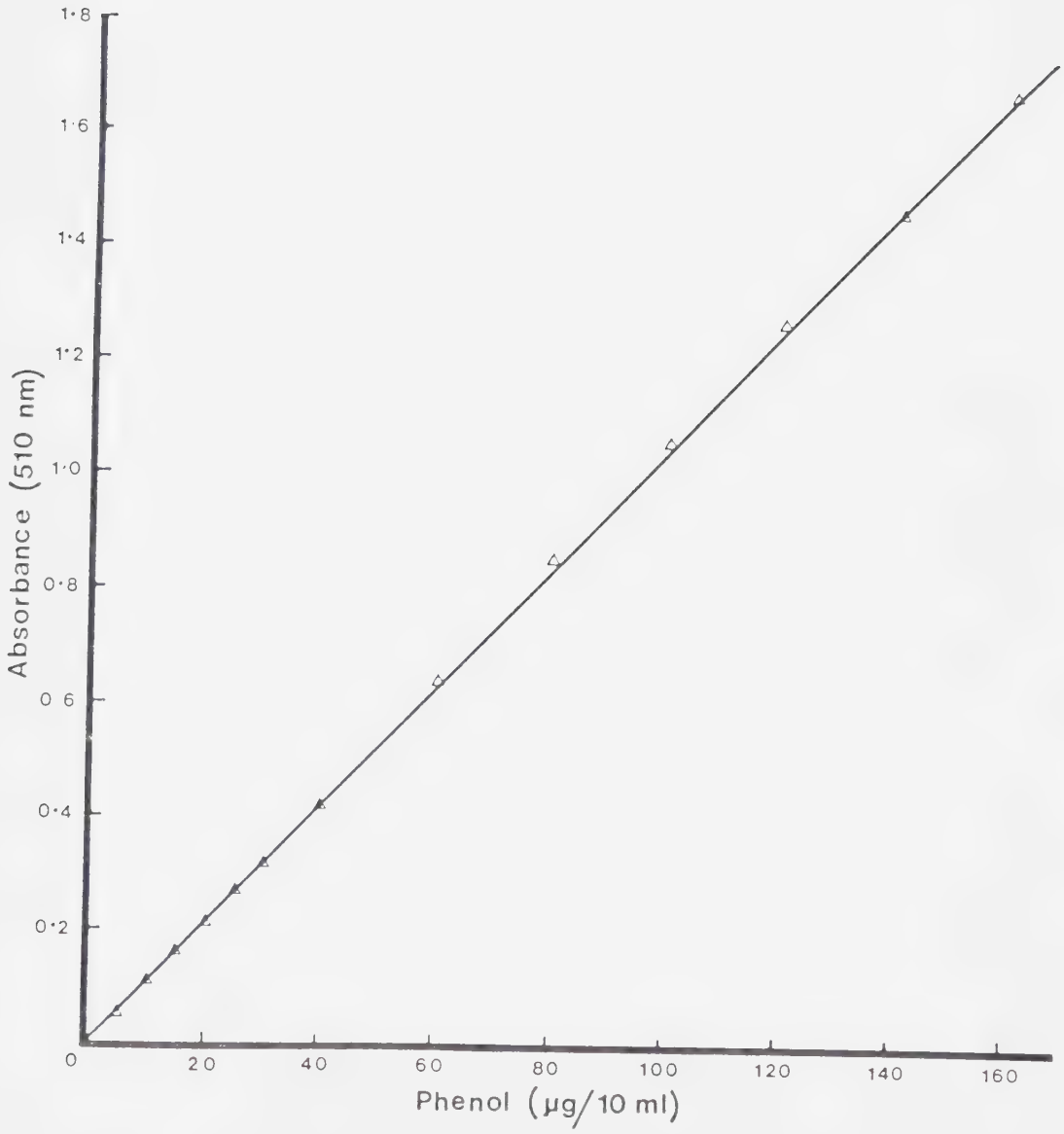


Figure 16. Calibration curve of standard phenol solution using the aminoantipyrine method.

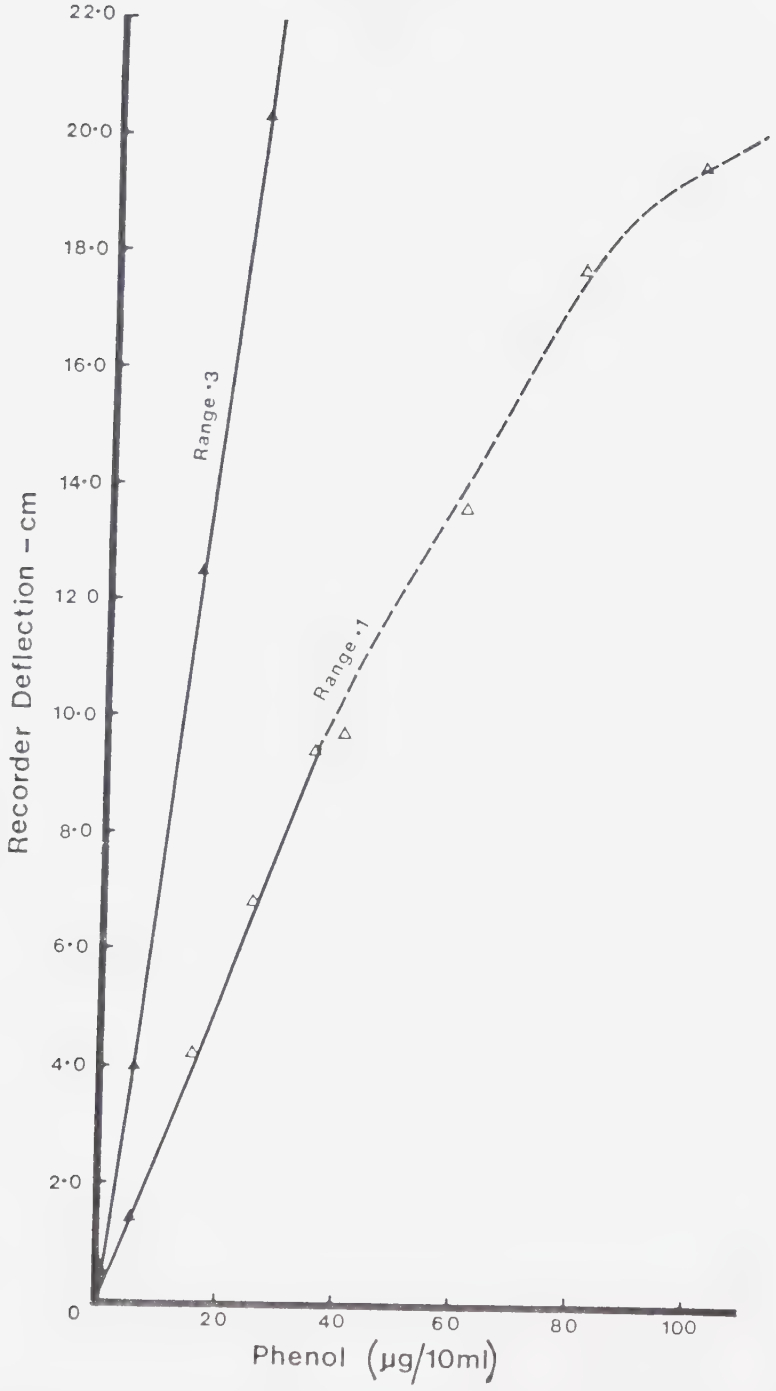


Figure 17. Calibration curve of standard phenol solution using fluorometric detection at range 0.1 and 0.3.

TABLE 5

Linear Regression Equations Showing Relationship between Phenol Actually Added (x) and Phenol Calculated (y) for Gibbs, Aminoantipyrine and Fluorometric Methods.

Method	Equation	Standard Regression Coefficient	Correction Factors[1]
Gibbs	y = 0.019766 x	1.000	50.592
Aminoantipyrine	y = 0.010551 x	1.000	94.778
Fluorometric	y = 0.257570 x	0.998	3.8824

[1]Calculated as inverse of slope (1/m)

fluorometric determination is plotted on two ranges of sensitivity, range .1 and range .3, respectively.

The recovery studies were necessary in order to calculate the volatility of phenol in honey and in wax. The studies involved the addition of known concentrations of phenol to honey and beeswax for each method and are presented in Tables 6-11. The data obtained from the spiking of honey were pooled together for the three methods, similarly for beeswax (Tables 12,13). The factors of 0.4267 and 0.6993 were derived for honey and wax analysis, respectively (corr. coeff. given in Table 14). The volatility of phenol in honey analysis was approximately 55% greater than phenol volatility in wax analysis.

The parameters listed in Table 15 were obtained after analysing the data from the calibration curves and recovery studies. For each of the methods, the linear relationship between the actual amount of phenol and the amount detected was greater for phenol in beeswax than it was for phenol in honey.

The calculation for quantitation of phenol in honey and wax samples is similar for the three methods. Two examples of calculations are shown for a honey sample determined by the Gibbs method, and a wax sample determined by the fluorometric method.

Phenol in honey (ppm) = A x 50.592^{1} x 0.4267^{2}

Phenol in wax (ppm) = recorder deflection(cm) x3.8824^{1} x 0.6693^{2}

A = absorbance measured at 610 nm.

1 = correction factor relating absorbance to ppm phenol (Table 5) and obtained from the calibration curve.

2 = volatility factor obtained from recovery studies (Table 14).

4.1.5 Investigation of Interferences in the Detection of Phenol

Investigation of possible interfering compounds arising from steam distillation of honey and perhaps, co-eluting with phenol was examined for the HPLC procedure on both normal and reverse phase columns by measuring the absorbance at several wavelengths. The results of analysis performed on both normal and reverse phase columns for honey samples were comparable. The

TABLE 6

Recovery of Phenol from Spiked Honey by the Gibbs Method.

Phenol Added (ppm)	Absorbance At 610 nm	Phenol Calculated (ppm)
1.0	0.049	2.5
4.0	0.183	9.1
6.0	0.280	14.2
8.0	0.383	19.3
9.0	0.425	22.0
10.0	0.447	23.0
15.0	0.664	34.0
20.0	0.936	48.0
30.0	1.310	67.0

TABLE 7

Recovery of Phenol from Spiked Beeswax by the Gibbs Method.

Phenol Added (ppm)	Absorbance At 610 nm	Phenol Calculated (ppm)
5.0	0.139	7.1
10.0	0.278	14.2
15.0	0.456	23.5
20.0	0.599	30.8
25.0	0.729	37.5
30.0	0.836	43.0

TABLE 8

Recovery of Phenol from Spiked Honey by the Aminoantipyrine Method

Phenol Added (ppm)	Absorbance At 510 nm	Phenol Calculated (ppm)
5.0	0.132	12.5
10.0	0.247	24.0
15.0	0.350	33.0
25.0	0.627	60.0
35.0	0.920	88.0
45.0	1.119	107.0

TABLE 9

Recovery of Phenol from Spiked Beeswax by the Aminoantipyrine Method.

Phenol Added (ppm)	Absorbance At 510 nm	Phenol Calculated (ppm)
5.0	0.110	10.0
10.0	0.207	20.0
15.0	0.295	28.2
25.0	0.450	43.0
35.0	0.626	60.0

TABLE 10

Recovery of Phenol from Spiked Honey by the Fluorometric Method.

Phenol Added (ppm)	Recorder Deflection[1] (cm)	Phenol Calculated (ppm)
1.0	0.8	3.0
5.0	2.8	10.3
8.0	5.1	19.0
10.1	6.1	22.4
15.1	9.2	34.0

[1]For range 0.1

TABLE 11

Recovery of Phenol from Spiked Beeswax by the Fluorometric Method.

Phenol Added (ppm)	Recorder Deflection[1] (cm)	Phenol Calculated (ppm)
1.0	0.7	3.0
5.0	2.2	8.0
8.0	3.5	13.0
10.0	4.2	15.0
15.0	5.9	20.1
30.0	10.0	37.0

[1]For Range 0.1

TABLE 12

Pooled Data Obtained from Colorimetric and Fluorometric Methods for the Determination of Volatility of Phenol in Honey.

Phenol Added (ppm)	Phenol Calculated (ppm)
1.0	1.62
4.0	8.40
6.0	13.31
8.0	18.52
9.0	20.64
10.0	21.75
15.0	32.73
20.0	46.49
30.0	65.42
1.0	2.68
5.0	10.72
8.0	19.37
10.1	23.06
15.1	35.10
5.0	12.51
10.0	23.41
15.0	33.17
25.0	59.43
35.0	87.20
45.0	106.10

TABLE 13

Pooled Data Obtained from Colorimetric and Fluorometric Methods for the Determination of Volatility of Phenol in Beeswax.

Phenol Added (ppm)	Phenol Calculated (ppm)
5.0	6.63
10.0	13.66
15.0	22.67
20.0	29.90
25.0	36.48
30.0	41.89
1.0	2.14
5.0	8.04
8.0	13.01
10.0	15.80
15.0	22.44
30.0	38.24
5.0	7.30
10.0	16.49
15.0	24.83
25.0	39.52
35.0	56.20

TABLE . 14

Factors Obtained from Recovery Data from Colorimetric and Fluorometric Methods for the Determination of Volatility in Honey and Beeswax.

Sample	Factor	Standard Regression Coefficient
honey	0.4267	0.999
beeswax	0.6693	0.997

TABLE 15

Parameters for Analytical Methods

Method of Analysis	Material Analysed	No. of Samples	Linear Region	Systematic Error	Correlation Coefficient
Gibbs	honey	9	0 to 30 ppm	+0.4 ppm	0.999
Gibbs	wax	6	0 to 47 ppm	+0.3 ppm	0.997
aminoantipyrine	honey	6	0 to 70 ppm	negligible	0.998
aminoantipyrine	wax	5	0 to 107 ppm	+2.1 ppm	0.999
fluorometric	honey	5	0 to 17 ppm	+0.2 ppm	0.999
fluorometric	wax	6	0 to 27 ppm	+0.4 ppm	0.995

variations obtained were consistent within limits of experimental error.

Possible interfering compounds from honey and wax were evaluated for the colorimetric and fluorometric assays by spiking a honey and wax at various levels and analysing the resulting data using linear regression analysis (Table 15). The systematic error represents interferences. In all cases, interferences are in a positive direction and are less than 0.5 ppm, except for the wax determination using the aminoantipyrine method. Possible interferences in the color reactions due to the presence of steam volatile phenolic compounds have been suggested by Dacre (1971) and in Standard Methods for the Examination of Water and Wastewater (1971). For fluorometric detection, interferences could be due to other steam volatile fluorescent compounds. It should be noted that only one honey and wax were spiked with phenol at various levels and used for the systematic error determination. One might expect, with the variety of honeys produced, that some samples could contain more interfering compounds than others.

4.1.6 Comparison of Analytical Methods

A comparison of the precision of the analytical methods is given in Table 16. These results and data from several honey and wax samples indicate the precision of the methods to be comparable. The fluorometric method is less precise than the HPLC and colorimetric methods.

The recommended method for phenol analysis in honey is by HPLC since it is rapid, simple and reliable. An important advantage of HPLC as compared to the other analytical methods is that quantitation is coupled with actual separation of the compound analysed. In an analysis peaks are identified using the retention times, which are consistently checked with that of the mixed standard.

The colorimetric tests using the Gibbs reagent and the aminoantipyrine reagent are not specific for phenol, but will react with all phenolic compounds, which may be present in the steam distillate of the honey. It would seem highly likely from a comparison of the results obtained, which show good agreement, that other steam volatile phenolic compounds are not present in significant levels

TABLE 16

Precision of Analytical Methods

Method	Number Of Replicates	Average Value Of Phenol (ppm)	Standard deviation
HPLC (4-chlorophenol internal standard)	5	7.9	0.10
aminoantipyrine (honey)	5	11.74	0.17
aminoantipyrine (wax)	3	2.17	0.12
Gibbs (honey)	5	3.66	0.09
Gibbs (wax)	5	3.02	0.19
fluorescence (honey)	7	9.70	0.27
fluorescence (wax)	4	6.15	0.49

or if they are, they do not react with the Gibbs reagent or aminoantipyrine reagent.

For laboratories without HPLC equipment, either colorimetric test, aminoantipyine or Gibbs may be used with the advantage that both honey and beeswax can be analysed. The aminoantipyrine method is quicker than the Gibbs test since the absorbance develops at room temperature and phenol levels can be monitored at a higher concentration. However, the aminoantipyrine method seems affected by interferences from beeswax to a greater extent. The linear region of the Gibbs reaction is limited by the concentration of the Gibbs reagent, but a higher concentration of reagent would result in a significant absorbance in the blank at the wavelength measured (610 nm) and increase the error, especially for samples containing smaller amounts of phenol. One advantage of the Gibbs method is that the absorbance can be measured over a period of 1 h because of the color stability.

The fluorometric method directly quantitates phenol from the steam distillate. However, it is less precise than the other tests and is limited by the range at which the concentration of phenol can be monitored. The fluorometric method is more subject to interferences from fluorescent compounds, apart from phenol in the steam distillate and in addition, may be quenched by other photochemical processes.

4.2 Analysis of Honey

The phenol residue levels of Albertan honeys are illustrated in the histogram (Figure 18). Levels of phenol quantitated for each honey are presented in Table 17. The numbers represent raw honey samples sent by individual beekeepers to the Alberta Honey Producers' Co-op. The Co-op accepts raw honey mainly from Alberta beekeepers, and also, from beekeepers from other parts of Western Canada for further processing and distribution.

Trace levels of phenol found in honey samples were typically 0.5 ppm or less. Contribution of phenol from the bee smokers could result in detectable traces of phenol (Lustre and Issenberg, 1969; Baltes *et al.*, 1981). Phenol levels

Figure 18. Distribution of Phenol Levels in Albertan Honey.

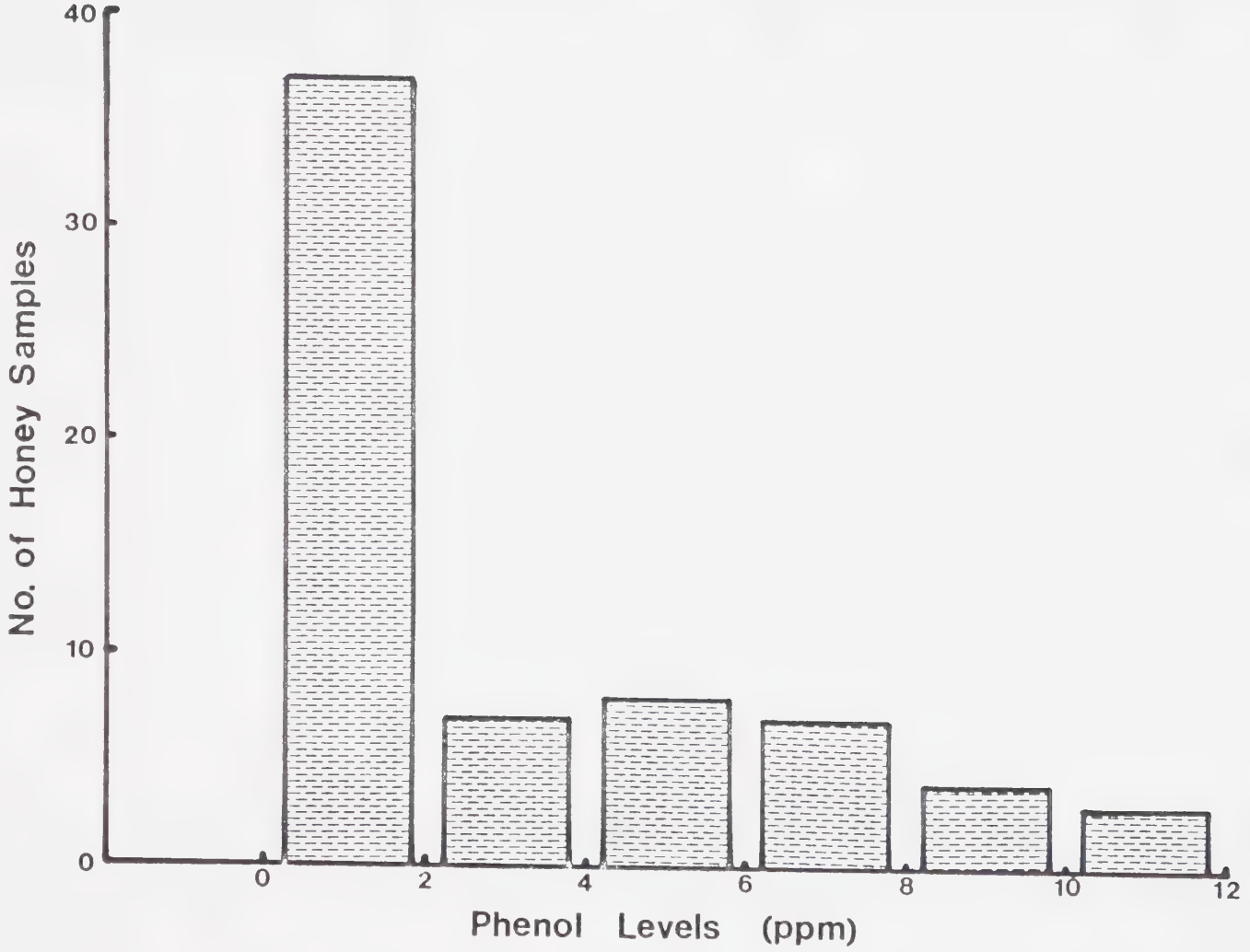


TABLE 17

Residues of Phenol Found in Albertan Honeys

Sample	Method Of Analysis[1]	Phenol (ppm)
556-5-80	Hnc - 269	5.0
246-6-44	"	8.5
4161-16-80	"	n.d.
243-64-80	"	4.8
602-12-80	"	n.d.
508-35-80	"	1.7
515-12-80	"	4.7
33-3-80	"	6.9
657-6-80	"	n.d.
580-16-80	"	8.1
513-18-80	"	4.2
601-8-80	"	4.9
500-2-80	Hnd - 269	n.d.
491-26-80	"	n.d.
632-59-80	"	tr.
517-6-80	"	n.d.
502-10-80	"	tr.
445-1-80	"	2.0
452-10-80	"	n.d.
540-32-80	"	10.0
530-5-80	"	5.9
516-26-80	"	n.d.
501-12-80	"	1.4
539-12-80	"	tr.
453-5-80	"	tr.

433-10-80	"	n.d.
343-27-80	Hnd - 269	n.d.
543-12-81	"	n.d.
101-5-81	"	n.d.
432-4-80	"	n.d.
348-11-80	"	n.d.
451-27-80	"	n.d.
455-2-80	"	n.d.
465-12-80	"	4.2
447-12-80	"	2.8
505-9-80	"	9.0
358-16-80	"	6.5
342-3-80	"	1.6
13-51-80	"	1.6
383-5-80	"	1.8
331-64-80	"	11.1
431-39-80	"	7.8
379-15-80	"	2.1
610-23-80	"	n.d.
360-10-80	"	n.d.
359-30-80	"	6.4
384-20-80	"	1.2
497-10-80	"	n.d.
357-4-80	"	6.3
248-4-80	Hrd - 195	1.0
304-64-80	"	3.8
497-10-80	"	n.d.
278-20-80	Hrd - 195	5.9
528-8-80	Hrc - 195	7.9
492-12-80	Hnd - 269	2.3

	Hnd - 195	2.2
	Hrd - 195	2.1
	A	2.4
	G	2.3
498-2-80	Hnc - 269	5.0
	Hnd - 195	4.1
	Hrd - 195	3.7
	A	3.4
	G	3.9
	F	3.6
509-10-80	Hnd - 269	4.3
	Hnd - 195	3.8
	Hrd - 195	3.5
	A	4.1
	G	3.7
292-65-829	Hnd - 269	3.7
	Hnd - 195	3.4
	Hrd - 195	3.3
	A	3.3
	G	3.7
443-24-80	Hnd - 269	1.3
	Hrd - 195	1.0
	A	1.1
	G	1.2
	F	1.1
528-8-80	Hnd - 269	7.5
	Hrd - 195	7.9
420-21-80	Hnd - 269	11.3
	Hnd - 249	10.1
332-23-80	Hnd - 269	4.5

	Hnd - 195	4.0
	Hrd - 195	4.0
	A	3.8
	G	3.7
	F	3.6
396-10-80	Hnd - 269	n.d.
	Hrd - 195	tr.
	A	tr.
	G	tr.
322-27-80	A	n.d.
	G	n.d.
606-66-80	G	1.2
600-24-80	A	10.8
	F	9.7
434-60-80	G	tr.
Individual rapeseed '81	Hnd - 269	6.5
Individual dandelion '81	"	12.8
Individual sweet clover '81	"	9.2
Individual sweet clover '81	"	n.d.
Commercial	Hnc - 195	2.6
Individual	Hnd - 269	24.0

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[1]Analysis Code

c = 4-chlorophenol, internal standard

269, 249, 195 = wavelengths monitored

d = 2,6-dimethylphenol, internal standard

r = reverse phase (octadecasilane coated silica) (HPLC)

H = HPLC method

n= normal phase column(HPLC)

A = Aminoantipyrine method

G = Gibbs method

F = Fluorescent method

n.d. = no phenol detected

tr. = trace

of 1 ppm or higher probably resulted from the use of phenol as a bee repellent. Thus, using 1 ppm as a cut off, 40 out of 67 samples of honey from the Alberta Honey Producers' Co-op or about 60% of the honey samples had been collected using a phenol board. The levels of phenol residue found in the 40 samples varied from 1 ppm to 11.1 ppm with an average of 7.4 ppm. Although the samples of honey from the Alberta Honey Producers' Co-op were held for at least six months at ambient temperature before they were supplied, analysis disclosed that significant amounts of phenol were retained in the honeys (later stored at $-22.5^{0}C$). Also, on examination of a honey sample from an individual beekeeper who had his honey rejected because of the off-taste, the level of phenol detected was 24 ppm and corresponded to the highest level quantitated for all honey samples. Analysis of this honey stored at ambient temperature over a period of three years revealed no detectable reduction in phenol concentration. These findings warrant the conclusion that phenol residue levels in honey are not significantly reduced with time. Phenol is, of course, steam volatile but removal of phenol would also result in loss of flavor volatiles, thus rendering the honey flavorless.

Table 17 and Table 18 list results of phenol levels in honey samples analysed by different methods and at different wavelengths to establish the possibility of interfering compounds in the quantitation of phenol. In all cases, it was found that the variation in the phenol residues of each honey analysed was minimal. Table 18 lists residue levels of phenol in honeys from other provinces in Canada and some foreign honeys including some from the United States. Honeys marked "individual" were labelled as being supplied by only one beekeeper. Honeys with floral sources given on the labels may or may not have been obtained from individual beekeepers. Honey marked "commercial" represented brand name honeys that are probably mixtures from many beekeepers and not necessarily beekeepers from the area in which the commercial honey was produced. From the limited data, it seems that the use of phenol as a bee repellent is relatively common in North America, but there is no evidence of the use of phenol in the samples of honey examined from

TABLE 18

Residues of Phenol Found in Other Honeys

Sample	Method Of Analysis[1]	Phenol (ppm)
Ontario, commercial	Hrc-195	1.7
B.C., individual	Hrd-195	tr.
B.C., orchard blossom	Hrd-195	6.0
B.C., alfalfa	Hnd-269	2.5
B.C., fireweed	Hnd-269	n.d.
B.C., wildflower	Hrd-269	4.3
Quebec, individual	Hrd-195	5.9
Quebec, individual	Hrd-195	2.3
	A	2.7
Florida orange blossom	Hrd-195	n.d.
Florida tropical wildflower	Hrd-195	11.2
	Hrc-263	12.6
	Hrc-269	12.6
	Hrc-275	13.3
	Hrc-195	12.6
	A	11.7
Washington State commercial	Hrd-195	tr
California clover	Hrd-195	tr.
California wildflower	Hrd-195	tr.
California sage	Hrd-269	n.d.
California clover	Hrd-269	n.d.
California safflower	Hrd-269	2.6

	A	2.1
Iowa commercial	Hrd-195	3.8
Yugoslavia forest	Hnd-269	n.d.
Yugoslavia sage	Hnd-269	n.d.
Yugoslavia meadow	Hnd-269	n.d.
Austrian commerical	Hnd-269	n.d.
Japanese commercial	A	tr.
Japanese commercial	A	n.d.
Mexican commercial	A	4.4
Mexican commercial	A	n.d.
Mexican commercial	A	3.0
China acacia	Hrd-195	tr.
Holland acacia	Hrd-195	tr.

[1]See Table 17 for analysis code.

other countries.

4.3 Field Trip

Residual levels of phenol in honey collected from four hives are presented in Tables 19-22. In the tables, "Time of direct exposure" is the time each super was exposed to the phenol board. Lower supers were exposed to the phenol board for the time given in the table plus the time of exposure for upper supers (at the extra distance 24 cm/super). Surface area of the frames collected is given by the number of impressions and the amount of material per unit surface area is given by the weight of material per impression.

Samples containing both honey and wax were stored for at least four days before analysis.

The data indicate that the phenol residues in honey are affected by several factors. The highest level (72 ppm, Table 19) was recorded for a honey removed from uncapped cells, with a small amount of material per unit surface area (19 g material/impression). This honey was also exposed for a long period of time (14 min plus upper exposure) at a short distance from the phenol board (6 cm) and at a very high temperature (approximately 48°C surface temp). Generally, an increase in phenol residue was associated with a higher surface temperature on the phenol board, greater exposure of the hive to the phenol board and closer proximity to the underside of the phenol board. In addition, samples collected from uncapped cells contained a higher proportion of phenol than material collected from capped cells.

In the case of capped cells it appeared that a rapid transfer of phenol occurred from the surface wax cappings to the honey underneath. Capped honey, sampled after the surface wax cappings were discarded (Tables 20-22) was found to have phenol residue levels similar to samples with cappings which were not discarded. Since it was only a matter of minutes, after exposure to the phenol board, before samples were collected, it seems that the penetration of phenol into the cells and the establishment of equilibrium between wax and honey are rapid.

TABLE 19

Results of Samples Collected from Hive 1 (separated and analysed 13 days after collection)

Super	Frame number (out of 9)	Honey cells	Time of direct exposure to phenol board (min)	Distance from top of frame (cm)	Number of impressions each (22.9 sq.cm)	Weight of material per impression (g)	Phenol board surface Temp. range (°C)	Weight of material collected (g)	Phenol in honey (ppm)
1[1]	5	U	20	11.0	3	19	35–44.5	58	18.6
2	5	U	6	15.0	3	23	44.5–45.5	69	12.5
	5	C		6.5	3	26		77	8.1
3	5	C	10	6.5	3	45	45.5–47.0	135.5	8.4
	9	C		6.5	3	33		100	6.8
4	5	C	14	6.0	3	32	47–49.0	96	13.9
	9	U		6.0	3	19		56	72.0
5[2]	5	C	19	6.5	3	30	49–50	89	12.1
	9	U		11.5	3	22		67	41.0

U = Uncapped, C = Capped, [1]Top, [2]Bottom

TABLE 20

Results of Samples Collected from Hive 2 (separated and analysed 4 days after collection).

Super	Frame number (out of 9)	Honey cells	Time of direct exposure to phenol board (min)	Distance from top of frame (cm)	Number of impressions each (22.9 sq.cm)	Weight of material per impression (g)	Phenol board surface Temp. range (°C)	Weight of material collected (g)	Phenol in honey (ppm)
1 (top)	5	M.C.	0	6.5	6	29	–	174	1.7
	5	U	0	19	3	17	–	51	0.6
1	4	C	27[1]	6	6	26	26–35Δ[2]	154	5.5
	4	C		17	5	28		139	2.8
	4	C, C.D.		6	6	21		127	3.5
	2	U		12	5	31		157	7.4
			5 mL more 90% phenol solution added to phenol board						
2	2	U	10	13	5	19	28.5–37.0	97	9.4
	8	C		6	5	38		192	5.3

	8	U		18	5	21		104	10.2
	8	C[3]		18	5	–		–	7.3
3 (bot)	2	C	6	6	6	32	37.0–38.0	190	9.4
	2	C,C.D.		15	5	24		121	2.3
	7	C		12	6	23		135	8.7
	7	C[3]		12	6	–		–	10.6

[1]Only 5 mL 90% phenol solution used

[2]Torch heating used

[3]Frames kept in honey house for 6 days before sampling and 2 days before analysis

M.C. = mostly capped C.D. = cappings discarded

TABLE 21

Results of Samples Collected from Hive 3 (separated and analysed 4 days after collection).

Super	Frame number (out of 9)	Honey cells	Time of direct exposure to phenol board (min)	Distance from top of frame (cm)	Number of impressions each (22.9 sq.cm)	Weight of material per impression (g)	Phenol board surface Temp. range (°C)	Weight of material collected (g)	Phenol in honey (ppm)
1	4	U	7	16	3	19	33.5–39	57	1.5
2	5	C,C.D.	11	13	6	30	39–40	179	5.2
	5	C		13	6	28		170	4.3
	1	C		8	5	27		134	9.1
3	4	U	7	13	6	13	40–45	77	14.2
	4	C		13	4	28		113	8.6
4	4	C	N.R.	7	6	35	43	208	14.0
		C		14	5	37		186	7.8

N.R. = not recorded

TABLE 22

Results of Samples Collected from Hive 4 (separated and analysed 4 days after collection).

Super	Frame number (out of 9)	Honey cells	Time of direct exposure to phenol board (min)	Distance from top of frame (cm)	Number of impressions each (22.9 sq.cm)	Weight of material per impression (g)	Phenol board surface Temp. range (°C)	Weight of material collected (g)	Phenol in honey (ppm)
1	4	U	0	O.E.F	14	4.4	–	62	0.8
1	4	U	17	O.E.F.	14	2.7	30–41	38	50.7
2	4	U	11	12	9	6.3	41–42	57	25.5
3	5	C,C.D.	28	13	6	15.0	42	88	6.4
	5	U		13	6	23.0		140	19.0
	4	C		15	6	24		143	8.4

O.E.F. = over entire frame

Separation of wax from a sample was performed by filtration and collection of the residue on cheesecloth. The residue, mainly wax was impossible to obtain totally free of honey. Analysis of the residue revealed a significantly greater concentration of phenol and the level was always higher than the separated honey. For example, a sample containing 6.2 ppm phenol in the honey had a level of 17.4 ppm phenol in the separated wax. Thus, the evidence presented supports the conclusion that honey from capped comb areas contains less phenol, at least in part, because the capping wax absorbs a large proportion of the phenol. The material collected from capped cells contained a higher proportion of wax than material from areas of uncapped cells. Certainly, the wax has a greater affinity for phenol. However, a greater surface area exposed by partially filled uncapped cells will account for higher phenol residues in uncapped comb.

The movement of phenol between wax and honey was also evident in samples unexposed to a phenol board (Tables 20 and 22, zero time of direct exposure). Honey collected already contained phenol. This phenol was almost certainly due to phenol migration from wax to honey later placed in the cells by the bees, since it is a common practice for beekeepers to reuse frames after collection of only the honey.

The results of several honey samples left in unsealed jars (Table 23) showed, that, within experimental error, there was no noticeable change in the phenol level of the honey, thus discounting the claims by Eckert (1962) that aeration of samples resulted in decreasing phenol levels. Phenol could be removed by steam distillation, but a flavorless honey would be obtained.

The examination of honey samples collected from frames after six days of storage in a beekeeper's honey house showed no substantial change in the phenol concentration compared to similar samples collected immediately (Table 20). It appears that the phenol levels in honey collected directly from the hives are unaltered by the transfer and storage in the honey house. The honey house is warmed and collected honey supers are stored before extraction of the honey to prevent crystallization of the honey in the combs.

TABLE 23

Effect of Aeration of Honey Samples.

Sample	Original Level Of Phenol In Honey (ppm)	Final Level Of Phenol In Honey (ppm)	Time Of Exposure Of Sample To Air (days)
1	10.2	9.9	10
2	1.7	1.6	11
3	3.5	3.3	11
4	2.8	2.6	11
5	5.5	5.8	11

Samples were sealed for seven months before honey and wax were separated and the honey analysed. As can be seen in Table 24, there is no change, within experimental error, in the phenol residues of the separated honey, indicating that no further change in the distribution of phenol had occurred after these samples were initially analysed.

Analysis of waxes was done on the sample amount available (Table 25). From the table it can be observed that a 2 g or 5 g weight sample gave the same result as from a 10 g sample.

In Table 20 one of the problems associated with the use of phenol is illustrated. When there is insufficient sunlight or air temperatures are low, the phenol is not volatilized enough to repel bees efficiently. The beekeeper in one case had heated the surface of the phenol board with a propane torch. This practice does not appear to cause a marked increase in phenol residues in the honey, provided the heating time is not excessive. The temperature recorded on the underside of the phenol board was approximately 50°C on heating with the propane torch. Under normal conditions the temperature on the underside of the phenol board was found to be about 10°C lower than the surface temperature recorded.

In the field trials, phenol boards were often left on the hive longer than required to repel the bees. To minimize phenol contamination of honey, it would be prudent for beekeepers to frequently observe the bees, so that phenol boards are in place, only as long as required. This would be especially important on warm sunny days. The beekeeper could also collect mainly capped honey to further minimize contamination.

Further evidence for the movement of phenol was obtained by analysis of samples of wax from a beekeeper who collected honey by use of the phenol board (Table 26). Samples of wax and honey from a beekeeper who had discontinued the use of phenol were examined for phenol residues (Table 27). The honey was found to be almost free of phenol after one year. Wax still contained small amounts of phenol, which could account for trace levels of phenol in the honey. Purchased foundation wax also contained small amounts of

TABLE 24

Phenol Levels in Honey Samples Before and After Storage

Sample	Original Level Of Phenol In Honey (ppm)	Phenol Level After 7 months (ppm)
1	5.2	5.2
2	5.3	5.6
3	1.7	1.9

TABLE 25

Analysis of Wax

Weight (g)	Phenol (ppm)	Method Of Analysis
2	2.6	Gibbs
5	2.6	Gibbs
10	2.8	Gibbs

TABLE 26

Analysis of Several Wax Samples

Sample[1]	Phenol (ppm)	Method Of Analysis
1	22.8	Gibbs[2]
2	42.1	Gibbs
3	22.0	Gibbs

[1]Samples obtained from a beekeeper who used a phenol board

[2]Duplicate samples analysed

TABLE 27

Analysis of Honey and Wax Samples Obtained from a Beekeeper.

Sample	Phenol (ppm)
Honey 1981[1]	3.3
Honey 1982[2]	trace
Wax comb 1981	1.2
New comb 1982	0.9
Foundation wax	1.7

[1]1981 samples collected using a phenol board.

[2]1982 samples collected without a phenol board.

phenol. Since foundation wax is recycled beeswax, it seems possible that low levels of phenol residues could appear in the honey of beekeepers who do not use phenol boards.

4.4 Sensory Evaluation

Experiments using the triangle test with untrained panelists are given in Table 28. Panelists were unable to distinguish between honey samples ($P<0.05$) and an increase in the phenol concentration of the honey did not appear to have the effect of increasing the number of correct responses. In these sessions of testing, several problems became apparent. Comments recorded by panelists indicated that reasons for differentiation included texture differences in samples (although they were handled in an identical manner except for phenol addition) and strong aftertaste. The strong aftertaste of phenol could certainly have made differentiation of samples difficult.

Subsequently, it was decided to use a different approach (Signal Detection Test) which incorporated the eating of a cracker and rinsing of the mouth with water in between samples to minimize aftertaste effects. In the Signal Detection Test, the panelists were asked to taste a control (no phenol) between each sample provided. The stimulus (phenol) presentation conformed to a series of approximately two-fold increases in concentration.

Table 29 summarizes the results obtained using the Signal Detection Test for the tasting of honey samples with the above precautions. It can be seen that there is a steady increase in discriminating ability as the phenol concentration increases. However, there was a large percentage of panelists indicating a difference between identical samples (R index = 46.4%).

Table 30 shows the data for the Signal Detection Test for phenol in water using the untrained panel. Panelists were able to discriminate samples from the reference (no phenol) at much lower phenol levels in water as compared to phenol levels in honey. It is interesting to note that the degree of discrimination is so dramatically affected by the sweetness of honey. The literature quotes several threshold taste levels of phenol detection varying form

TABLE 28

Taste Results of Phenol in Honey Using the Triangle Test

Levels of phenol in honey compared	No. Of Panelists Correctly Identifying Odd Sample Over Total Trials	Probability[1] That Samples Were Correctly Chosen At random
0 vs 5.2	5/10	0.213
0 vs. 14.8	9/30	0.714
0 vs. 18.7	6/18	0.588
0 vs. 28.6	6/20	0.703

[1]Roessler et al., 1978.

TABLE 29

Results of Signal Detection Test for Phenol in Honey.

Phenol (ppm)	R index[1] (%)
0.0[2]	46.4
6.9	58.3
13.0	65.0
27.0	70.6

[1]O'Mahony, 1979.

[2]Identical to reference sample.

TABLE 30

Results of Signal Detection Test for Phenol in Water.

Phenol (ppm)	R index (%)
2.2	87.8
5.3	89.8
11.3	88.0

0.0125 ppm in beer, 0.01 ppm in butter to a report of 25.0 ppm in water. These tests were mainly carried out using trained panelists (Section 2.5).

For quantitation of phenol in honey instrumental methods of analysis are, of course, superior. However, to establish a definite threshold level of phenol in honey would require considerable time and effort to train panelists. Also, conclusive results can be established only if problems with texture, lingering aftertaste, and sweetness interference can be reduced. Furthermore, with the diversity of honey flavors there are probably a variety of threshold levels.

The entire question of threshold levels of phenol in honey may be only of academic interest if future government regulations eliminate the use of phenol as a bee repellent.

5. CONCLUSIONS AND RECOMMENDATIONS

Samples of honey and beeswax were examined for residual levels of phenol. The honey samples were chiefly from Alberta and were supplied by the Alberta Honey Producers' Co-op which accepts raw honey, mainly, from beekeepers from Alberta and also from beekeepers from other parts of Western Canada for processing and distributing. Analysis of the samples revealed that phenol is widely used in Alberta and other Canadian provinces. Phenol residues were also found in honey samples obtained from the United States and Mexico.

The major factors affecting the residual phenol levels were practically investigated by application of phenol on a phenol board. The 90% (w/v) phenol solution was highly effective as a repellent under sunny conditions or when the surface temperature of the phenol board was above 28°C. It was observed that the high efficiency of phenol as a repellent resulted in rapid collection of honey. However, the use of phenol always resulted in residues of phenol in honey. Thus, an essential precaution in the use of phenol requires careful observation by the beekeeper to minimize exposure of honey, since contamination can easily occur. Uncapped honey samples contained higher levels of phenol than capped honey samples. Examination of beeswax showed that it was also necessary to monitor phenol residues since wax has a greater affinity for phenol than honey. This is especially important since empty frames are reused for seasonal collection of honey. Also, foundation wax is recycled beeswax and analysis of honey unexposed to a phenol board contained low residual levels of phenol indicating phenol transfer to wax and subsquent migration to honey, later produced by bees in the reused frames. Further studies are required to determine the partition coefficient of phenol in honey and wax.

The procedures formulated for the detection of phenol in honey and wax proved to be very efficient and reliable. The HPLC method of analysis on the reverse phase column is highly recommended because of the accurate quantitation which also involved separation and identification of phenol. For the

HPLC method the internal standard, 4-chlorophenol proved to be highly suitable. Monitoring of the absorbance at 195 nm resulted in greater sensitivity than at 269 nm. Quantitation of beeswax by the colorimetric tests proved to be quite satisfactory. Extraction of the phenol by steam distillation was a prerequisite step for the isolation of phenol from honey or beeswax for the HPLC method, colorimetric and fluorometric techniques.

The untrained panelists showed greater discriminating ability in detecting phenol in water than in honey. Further studies in sensory evaluation are necessary to establish a taste threshold in a particular honey, since the detection of phenol is complicated by factors such as sweetness, texture and lingering aftertaste.

Other possibilities that could be examined are the monitoring of residual levels of other bee repellents such as propionic anhydride, butyric anhydride and benzaldehyde. It would be interesting to monitor for residues of benzoic acid since the oxidation of benzaldehyde to benzoic acid, as claimed by two authors seems to be a remote possibility. Further investigation of pheromones could provide a potential bee repellent. Since honey is prided for its natural flavor, research in developing a noiseless bee blower might provide a quick solution in the collection of honey without contamination of honey by chemicals.

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APPENDIX 1

QUESTIONNAIRE FOR TRIANGLE TEST

Name: _____ Date: _____

Test: _____

Two of these three samples are identical, the third is different.

1. Taste the samples in the order indicated and identify the odd sample.

Code	Check Odd Sample
_____	
_____	
_____	

2. Indicate the degree of difference between the duplicate samples and the odd sample.

Slight _____

Moderate _____

Much _____

Extreme _____

3. Describe the type of difference between the duplicate and the odd sample.

APPENDIX 2

THRESHOLD STUDY

Experiment: _____

Name: _____ Date: _____

Compare each test sample with the reference sample marked "R" Determine if the test samples are different or not different from the reference sample and the degree of certainty of your decision (sure, not sure). You must make a decision in this study. Taste the test samples in the order presented.

	Different		Not Different	
SAMPLE	Sure	Not Sure	Sure	Not Sure

Comments :